PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 31/18, 31/185, 31/19
A61K 31/255, 31/215, 31/795
C07C 309/43, 309/44, 310/04
C07C 311/16, 317/22

A1

(11) International Publication Number: WO 92/12708

(43) International Publication Date: 6 August 1992 (06.08.92)

(21) International Application Number: PCT/US92/00501

(22) International Filing Date: 21 January 1992 (21.01.92)

(30) Priority data:

647,469	29 January 1991 (29.01.91)	US
647,720	29 January 1991 (29.01.91)	US
792,135	13 November 1991 (13.11.91)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTI-COAGULANT PROPERTIES OF MACROCYCLIC COMPOUNDS AND METHOD OF TREATMENT

(57) Abstract

A pharmaceutical composition is disclosed for use in inhibiting blood coagulation in a human subject. The composition includes a macrocyclic compound composed of aryl ring subunits connected one to another by ring-attached bridge linkages, and containing sulfonic acid-derived substituents carried on non-bridge atoms of the subunits, carried in a pharmaceutically acceptable carrier. Also disclosed are novel macrocyclic compounds.

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ANTI-COAGULANT PROPERTIES OF MACROCYCLIC COMPOUNDS AND METHOD OF TREATMENT

This invention is a continuation-in-part of U.S. patent applications for "Macrocyclic Anti-Viral Compound and Method", Serial No. 647,720, and "Method of Treating Herpes Simplex Viral Infection, Serial No. 647, 469, both filed January 29, 1991.

1. Field of the Invention

The present invention relates to macrocyclic anticoagulant compounds and methods of inhibiting blood coagulation.

2. References

Andriuofi, G. et al. (1990) Hemostasis 20 (suppl. 1): 154-158.

Baboir, B.M. and Stossel, T.P. (1984) Hematology: A Pathophysiological Approach. Churchill Livingstone, New York, pp.171-189.

Brown, B.A. (1988) Hematology: Principles and Procedures. Lea & Febiger, Philadelphia, pp. 204-254.

Caramazza, I. et al. (1991) Thrombosis Research 62:785-789.

Majerus, P.W. et al. (1990) in Goodman and Gilman's The Pharmaceutical Basis of Therapeutics (Gilman, A.G.

et al., eds.) Pergamon Press, New York, Chap. 55: 1311-1331.

Powers, L.W. (1989) Diagnostic Hematology: Clinical and Technical Principles. C.V. Mosby Co. St. Louis, pp. 135-143.

3. Background of the Invention

Blood coagulation or clotting is the result of a complex series of biochemical reactions. In the normal course of events, hemostasis and the associated process of blood coagulation prevent undue loss of blood from an injured blood vessel. However, inappropriate coagulation of blood may occur within the circulatory system in pathological states such as atheroschlerosis or in response to a variety of insults, including surgery and implantation of medical devices. This can lead to occlusion of a vessel and/or thromboembolism, in which all or part of a blood clot breaks loose and becomes lodged as an embolus in another region of the circulatory system. Such emboli are, in some cases, life-threatening, especially when they cause obstruction of the pulmonary or cerebrovascular circulatory system.

Prevention of blood clotting is therefore considered a crucial part of the treatment regimen for patients at risk for developing thromboemboli. Disease or treatment states in which anticoagulant therapy is indicated include replacement of heart valves, grafting procedures, chronic bedrest, surgery, venous thrombosis and pulmonary embolism, arterial embolism, stroke, presence of abnormal coagulation factors, certain stem cell diseases, and homocystinuria.

In order to understand the various means by which blood coagulation can be controlled, a basic understanding of the cascade of reactions leading to formation of fibrin and blood clots within the

circulatory system is essential. These reactions and their components have been reviewed extensively (Majerus,Baboir) and will be only summarized briefly with reference to Figure 14 herein.

Coagulation of blood can be stimulated by either of two different, but interconnected pathways - the intrinsic and extrinsic pathways. In both pathways, blood coagulation results from a series of zymogen activation steps involving enzymatic cleavage of the inactive zymogen molecule to an active protease, which, in turn, activates the next enzyme in the pathway. With reference to Figure 14, the linking point between the intrinsic and extrinsic pathways is activation of the zymogen Factor IX to the active protease, Factor IXa.

The intrinsic pathway is so called, because, following the initial contact stimulus, only factors intrinsic to the blood are involved in its functioning. In this pathway, as studied in vitro, interaction of Factor XII, prekallikrein, and high molecular weight kininogen with a foreign surface, such as glass or kaolin, results in conversion of Factor XI to Factor XIa, which activates Factor IX to Factor IXa.

Factor IXa is a protease which converts inactive Factor X to active Factor Xa. This conversion is accelerated by the presence of platelets or phospholipids (both designated PL in the figure), cofactor VIIIa, and calcium. The conversion of Factor II (prothrombin) to form Factor IIa (thrombin) is enhanced by the presence of platelets or phospholipids, factor Va, and calcium. Factor Va can be released by stimulated platelets.

Thrombin is a protease which cleaves the high molecular weight fibrinogen to fibrin monomers. These monomers form a gel, to which red blood cells adhere to form a blood clot. The strength of the clot is increased

by the fibrin monomer interchain transglutamination reactions, catalyzed by factor XIIIa.

To complete the common pathway shown in figure 14, clots are broken down ("dissolved") by an endogenous fibrinolytic system. The active protease plasmin is formed from inactive plasminogen by enzymatic cleavage catalyzed in vivo by one or more of a number of endogenous activators, including tissue plasminogen activator (t-PA). Streptokinase, a bacterial product, or urokinase, isolated from human cells, are also capable of activating plasminogen, as shown in figure 14. Plasmin non-specifically cleaves fibrin and other plasma proteins, including some of the clotting factors.

In the extrinsic pathway, exposure of blood to a tissue factor is the stimulus for conversion of Factor IX to Factor IXa. Tissue Factor is a lipoprotein present on surfaces of non-circulatory cells, such as fibroblasts, or activated monocytes or endothelial cells to which the blood may be exposed in certain pathological states. As shown in Figure 14, Factor VIIa, in the presence of calcium, effects the conversion of Factor IX to Factor IXa as well as the conversion of Factor X to Factor Xa. Factor VII itself has about 1/100 the proteolytic activity of Factor VIIa, and is therefore able to initiate clotting. Tissue factor increases the activities of both Factor VII and Factor VIIa about 30,000 fold. Formation of Factor Xa, also accelerates the process by converting still more Factor VII to Factor VIIa.

In general, agents which affect blood hemostasis fall into three categories: agents which interfere with platelet activation and aggregation, agents which interfere with portions of the above-described coagulation cascade, and agents which promote disintegration of blood clots. Aspirin, dipyridamole

and ticlopidine are examples of anti-platelet drugs. Their utilities as anti-clotting agents are generally limited to prophylaxis against atherosclerotic disease, repeat myocardial infarction, transient ischemic attack, and alone or in association with anticoagulants in certain cardiac valvular disorders. They are not generally used in the treatment of other abnormal clotting events, such as venous thrombosis, nor is there considered to be a mechanistic basis for their use in such disorders.

Agents which promote disintegration of blood clots (fibrinolytic agents) include tissue plasminogen activator, streptokinase and urokinase. These compounds are used post-myocardial infarction to prevent thromboembolism.

Currently available anticoagulant drugs are limited to the heparin-like compounds, which are active only when given intravenously, and to the oral coumarin anticoagulants. Heparin is an endogenous glycosaminoglycan which serves as a catalyst for the reaction between antithrombin and various activated factors in the coagulation cascade (Factors IXa, Xa, XIa, XIIa, kallikrein and thrombin). This reaction results in inhibition of these factors, and thus inhibition of coagulation. Heparin is not well absorbed orally and has a relatively short half-life in the bloodstream. Side effects of long term heparin therapy can include thrombocytopenia with associated paradoxical arterial thrombosis, and, rarely, osteoporosis. Overdosage with heparin can be antagonized by injection of protamine sulfate.

Oral anti-coagulants, including warfarin and other coumarin derivatives, produce their effects on blood coagulation by indirect means. These compounds inhibit regeneration of vitamin K in the liver. Vitamin K is a

precursor to several of the coagulation pathway factors, including Factors II (prothrombin), VII, IX, and X; therefore, depletion of vitamin K results in inhibition of coagulation. As might be expected from their mechanism, the coumarin drugs have a relatively long onset of therapeutic activity, since their effectiveness is dependent upon depletion of endogenous depots of active vitamin K. Coumarin therapy requires careful management, due to a number of drug and nutritional interactions which serve increase or decrease effective dosage levels. Treatment with coumarin derivatives is also associated with several serious side effects including bleeding episodes and teratogenicity.

A number of analytical tests have been devised to measure the patency of the above-described coagulation cascade. These tests, which are described in more detail below, are generally carried out on blood plasma. For example, the prothrombin time assay (PT) is measures the extrinsic system of coagulation and is therefore used to detect deficiencies in factors II, V, VII, and X. PT is also used to monitor therapy in patients receiving coumarin anti-coagulants, since factors II and VII are among those which are dependent upon vitamin K.

The activated partial thromboplastin time assay (APTT) measures coagulation factors present in the intrinsic system of coagulation, with the exception of platelets and factor XIII. It is generally used to monitor heparin therapy. Plasma clotting time is another measurement of the intrinsic coagulation pathway, and is also useful in monitoring heparin therapy.

As described above, current anti-coagulant regimens include treatment with various forms of heparin, or coumarin drugs. Of the two, the heparin drugs are by far the better tolerated and are easier to titrate. However,

the usefulness of these compounds is limited by their currently obligatory intravenous route of administration. Although formulations of these compounds have been administered enterally, anticoagulant activity has been observed only after intraduodenal administration (Andriuoli, Caramazza).

Coumarin drugs such as warfarin can be given orally; however, the usefulness of these drugs is limited by their relatively long onset time, difficulty in titration, interactions with other drugs, and side-effects, as described above. It is therefore a general object of the present invention to provide compounds and methods for oral anti-coagulant therapy with a shorter onset and duration of action, for improved oral control of blood coagulation.

4. Summary of the Invention

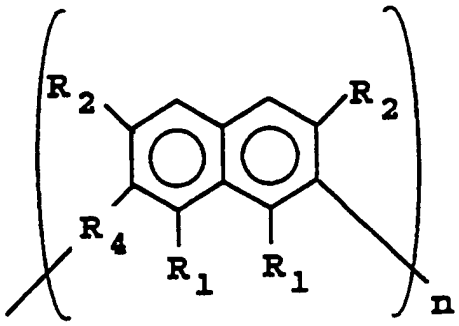
It is a general object of the invention to provide a compound and method effective to treat blood coagulation pathologies in a human subject.

The present invention provides a method for inhibiting blood coagulation. The method involves administering to a subject a macrocyclic compound composed of aryl ring subunits connected one to another by ring-attached bridge linkages which form a continuous chain of connected backbone atoms. The subunits have sulfonic acid-derived substituents on non-backbone atoms of the aryl subunit rings.

The ring subunits preferably include naphthalene subunits with sulfonic-acid derived substituents at the 3 and 6 ring positions, phenyl subunits with sulfonic acid-derived substituents at the 4 ring position, where the bridge linkages in the macrocycle are between the 2 ring-carbon position of one naphthalene or phenyl group, and the 7 ring-carbon group of an adjacent naphthalene group

or 6 ring-carbon position of an adjacent phenyl group. The compound preferably includes 4-8 such subunits. The sulfonic acid-derived substituent is preferably sulfonic acid, a sulfonate salt, sulfinic acid, a sulfinate salt, a sulfone, or a sulfonamide.

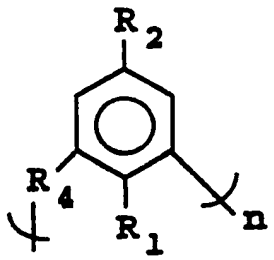
In one general embodiment, the macrocyclic compound includes at least 4 naphthalene subunits, each having sulfonic acid-derived substituents at 3 and 6 ring-carbon positions, polar groups at 1 and 8 ring positions, and bridge linkages between the 2 ring-carbon position of one subunit and the 7 ring-carbon position of an adjacent subunit. One preferred compound of this type has the general structure:



where R_2 is sulfonic acid, sulfonate salt, sulfinic acid, a sulfinate salt, an alkyl sulfone, or a polar sulfonamide, such as SO_2NHR, where NHR is NH_2, NHOH, or an amino acid, R_1 is OH, =O, an alkyl or aryl ether, ester, or acid, or a mixture thereof, and n = 4, 6, or 8, and R_4 is >CHR" or ≥CR", where R" is H or carboxylic acid group.

In another general embodiment, the macrocyclic compound includes at least 4 phenyl subunits with para-position sulfonic acid derived substituents, bridge linkages between the 2 ring-carbon position of one

subunit and the 6 ring-carbon position of an adjacent subunit. One preferred compound of this type has the general structure:



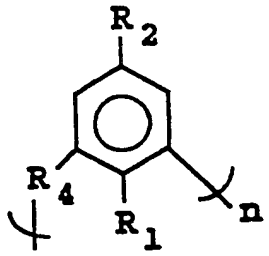
where n, R_1, R_2 and R_4 are as above.

For use as an anticoagulant, the compound may be administered orally or parenterally. Such treatment method may further include administering to the subject a dose of protamine sufficient to reverse anti-coagulant effects of the compound.

In another aspect, the invention includes a method of inhibiting blood coagulation by administering to the subject a therapeutically effective dose of a macrocyclic biocompatible polymer composed having at least six regularly spaced sulfonic-acid derived substituents selected from the group consisting of an alkyl sulfone, and a polar sulfonamide of the form SO_2NHR, where NHR is NH_2, NHOH, or an amino acid.

In a preferred embodiment, the polymer is a macrocyclic compound composed or aryl ring subunits which are connected by ring-attached bridge linkages which form a continuous chain of connected atoms making up the backbone of the macrocycle, and which contain the sulfonic-acid derived substituents on non-backbone atoms of the aryl subunits.

Also forming part of the invention are novel macrocyclic compounds composed of phenyl subunits having the form:



where R_2 is sulfonic acid, sulfonate salt, sulfinic acid, sulfinate salt, a sulfone, or a sulfonamide, R_1 is =O, and -OH, an alkyl or aryl ether, ester, or acid, or a mixture thereof, R_4 is >CHR" and >CR", where R" is H or carboxylic acid group and n = 4, 6, or 8.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the general structure of a macrocyclic compound composed of naphthalene subunits, for use in the present invention;

Figures 2A and 2B show non-oxidized (2A) and partially oxidized (2B) forms of the Figure-1 structure, where n=4 and the subunit is chromotropic acid;

Figures 3A and 3B illustrate two general methods of synthesis of a macrocyclic compound like the one shown in Figure 2A;

Figures 4A and 4B show an unoxidized (4A) and partially oxidized (4B) macrocycle with mixed phenyl and sulfonated naphthalene subunits;

Figure 5 illustrates reaction methods for converting the sulfonic acid substituents of macrocyclic

chromotropic acid to glycyl sulfonamide and sulfonamide groups;

Figure 6 illustrates a reaction method for converting the sulfonic acid residues of macrocyclic chromotropic acid to sulfinic acid or its methyl (aryl) ester;

Figure 7 shows the general structure of a macrocyclic compound composed of phenyl groups with para-position sulfonic acid-derived substitutents, for use in the present invention;

Figure 8 shows a non-oxidized form of the Figure-7 structure, where n=4 and the subunit is parasulfonic acid;

Figures 9A and 9B illustrate general methods of synthesis of non-oxidized and partially oxidized forms of the Figure 8 compound;

Figure 10 shows a reaction scheme for replacing the ring hydroxyl groups in the Figure-8 compound with acetyl groups;

Figure 11 shows a reaction for converting sulfonic acid substituents to a glycyl sulfonamide group in a phenyl-subunit macrocyclic compound;

Figure 12 shows a reaction scheme for producing a macrocylic compound like that shown in Figure 8 but with carboxylic acid-containing bridge linkages;

Figure 13 shows a reaction scheme for replacing hydroxyl groups in the Figure-8 compound with carboxylic acid groups.

Figure 14 shows a schematic of the cascade of biochemical events which occur in the coagulation process in mammalian blood;

Figure 15 shows a plot of prothrombin time in seconds (PT) as a function of concentration of KY-1, Y-1, and Y-49;

Figure 16 shows a plot of activated partial thromboplastin time in seconds (APPT) as a function of concentration of KY-1, Y-1, and Y-49;

Figure 17 shows a plot of APTT determined at various times after intravenous injection of 2.5 mg/kg (X) or 5.0 mg/kg (Δ) Y-1 in mice, where APTT is expressed as percent untreated control sample value run in parallel;

Figure 18 shows a plot of percent control APTT as a function of i.v. injected dose of Y-1 in rats, where APTT is expressed as percent untreated control value run in parallel;

Figure 19 shows a plot of thrombin time (TT) as a function of concentration of KY-1, Y-1, and Y-49;

Figure 20 shows a plot of atroxin time (AT) as a function of concentration of KY-1, Y-1, and Y-49;

Figures 21 (A-G) show traces of change in optical density as a function of time in a platelet aggregation assay in which platelet aggregation was measured in the presence of collagen (A), collagen plus 24 (B) or 48 μg/ml (C) Y-49, collagen plus 24 (D) or 48 (E) μg/ml Y-1, collagen plus 24 (F) or 48 (G) μg/ml KY-1;

Figures 22 (A-B) show plots of effects of varying concentrations of Y-1 (A), KY-1 and heparin (B) on plasmin activity;

Figure 22C is a bar graph summarizing the data of Figures 22A and 22B (C8 = Y-1);

Figure 23 A shows a composite plot of varying concentrations of KY-1 on clotting times measured as PT, TT, APTT, and AT;

Figure 23B shows a composite plot of varying concentrations of Y-1 on clotting times measured as PT, TT, APTT, and AT; and

Figures 24A and 24B show plots of TT and APTT as a function of concentration of KY-1 (24A) or heparin (24B) concentration in plasma.

Detailed Description of the Invention

I. Definitions

The terms defined in this section have the following meanings unless otherwise indicated.

"Anticoagulant activity" refers to the inhibition of the normal blood coagulation or clotting process, exhibited as prolongation of the time required to form a fibrin aggregate or clot, as measured by one or more standard in vitro clotting assay.

An "aryl ring" subunit is single ring or fused ring structure containing at least one aromatic ring, i.e., a 5- or 6-membered ring with the 6 pi electrons necessary for aromaticity. Examples include benzene, naphthalene, mixed aromatic and non-aromatic fused ring structures, such as tetralin, and heterocyclic structures, including fused-ring structures, such as quinoline, isoquinoline, and indole.

A "macrocyclic compound composed of aryl ring subunits" is a cyclic compound formed by linking ring atoms in aryl ring subunits to form a cyclic chain.

A "ring-attached bridge linkage" is a linkage between a ring atom of one aryl subunit to a ring atom of an adjacent aryl subunit in a macrocyclic compound;

The ring-attached bridge linkages and the (shorter path of) ring atom joining bridge linkages in the subunits collectively form a "continuous chain of connected backbone atoms". In the compound illustrated in Figure 1, the chain is formed by the bridge linkages (R_4) to positions 2 and 7 of the naphthalene rings and the 5 ring atoms in naphthalene between positions 2 and 7. In the compound illustrated in Figure 7, the chain is formed by the bridge linkages (R_4) to positions 2 and 6 of the benzene rings and the 3 ring atoms in benzene between positions 2 and 6.

Similarly, the "non-chain ring atoms" in the macrocycle are the ring atoms which are outside the bridge linkages. In the compound illustrated in Figure 1, the non-chain atoms include the 5 naphthalene ring atoms from ring positions 3-6; in the Figure-7 compound, the 3 ring atoms from positions 3-5.

A "sulfonic acid-derived substituent" includes sulfonic acid, a sulfonic acid salt, sulfinic acid, sulfinate salts, alkyl and aryl sulfones, sulfonamides of the form SO_2NHR, where R is H or a substituent having an OH, ether, ester, ketone, or acid moiety.

II. Preparing Aryl-Subunit Macrocyclic Compounds

This section describes the synthesis of two general types of aryl macrocyclic compounds which are useful in the anti-viral treatment method of the invention. The first type is composed of naphthalene subunits with sulfonic acid-derived substituents, described in subsection A. The second general type is composed of phenyl subunits having para-position sulfonic acid-derived substituents, described in subsection B. From the synthetic routes given in the two sections, it will be apparent how macrocycles composed of mixed subunits, e.g., both naphthalene and phenyl subunits can be prepared. The synthetic methods are also generally applicable to macrocycles composed of heterocyclic subunits with sulfonic acid-derived substituents.

A. Macrocyclic Compounds with Substituted Naphthalene Subunits

Figure 1 shows the general structural formula of a macrocyclic compound composed of substituted naphthalene subunits, for use in the present invention. One exemplary compound of this type is shown in non-oxidized (I) and partially oxidized (II) form in Figures 2A and

2B, respectively. The compound is a tetramer of chromotropic acid (1,8-dihydroxy, 3,6-disulfonic acid naphthalene) subunits linked by methyl or methylene ($>CH_2$ or $\geq CH$) bridges (R_4). As seen, the methylene bridges and the "interior" ring atoms (ring positions 1, 2, 7, and 8) form a continuous chain having attached R_1 = OH or =O groups attached at the 1 and 8 positions. The non-chain atoms (ring positions 3-6 on each substituent) have R_2 = sulfonic acid substituents on the 3 and 6 ring atoms. The nature of the partially oxidized structure was deduced from H^1 and C^{13} NMR studies, and from mass spectroscopy evidence.

For purposes of the following discussion, and for illustrating synthetic routes, usually only the non-oxidized subunit form of the compound is given. It will be understood that the compound may be partially oxidized, after exposure to air under heat and acidic conditions, i.e., contain one or more R_1 ketone (=O) groups, and a double bond between the ring and the associated bridge methylene group, as indicated in Figure 2B. It will also be understood that the same reaction mechanisms will apply generally to the partially oxidized form of the compound, i.e., the structure shown in Figure 2B, or similar structures containing additional R_1 =O groups, except that R_1 modification reactions will typically selectively modify an R_1 -OH group, and leave the corresponding R_1 =O group intact.

As will be seen below, the compound preferably includes the chromotropic acid derivatives in which R_1 is a polar substituent, such as OH, =O, CO_2H or an ether, thioether, ester, or thioester linked alkyl or aryl group, and combinations of these group, e.g., where only the OH groups in the partially oxidized structure are substituted by one of the above groups.

R_2, as noted, is a sulfonic acid-derived substituent which may be sulfonic acid, as shown in Figure 2, a sulfonate salt, sulfinic acid ($-SO_2H$), and sulfinate salts, sulfones, and sulfonamides. R_3 is H or Br or other halogen. Also as will be seen below, the R_4 bridge linking the chromotropic acid derivative subunits is preferably of the form >CHR or ≥CR (indicating unsaturated bridges in the partially oxidized form), where R is H or a small carbon-containing group, such as lower alkyl, alkenyl, ketone, or carboxylic acid group, or aryl group. The bridge may also be of the form $-CH_2NR'CH_2-$, where R′ is similarly H or a small carbon containing group, such as a lower alkyl group.

Alternatively, the bridges in the macrocycle may be ring structures, including aryl ring structures, such as in the dimeric macrocycle shown in Figure 4, or analogous structures formed by bridging through heterocyclic rings, such as pyrole or furan rings.

The number of subunits may vary from 4 to 8, with macrocycles containing 4, 6, and 8 subunits being preferred. In the reaction schemes described below, the macrocycle formed may include mixtures of compounds with different subunit numbers (n) values, e.g., a dominant n=4 structure (4 subunits) with additional structures containing 6 and 8 subunits.

Representative macrocyclic compounds which have been synthesized and tested for anti-viral activity are identified by their R_1, R_2, R_3, and R_4 substituents in Table 1 below. The KY and Y number in the lefthand column in the table refers to the analog designation of the corresponding compound. For example, the compound in which R_1 is OH, R_2 is SO_2NH_2, R_3 is H, and R_4 is $-CH_2-$ is designated KY-3.

Although not shown in the table, the compounds may exist in a partially oxidized state in which one of more R_1

groups are =O, and adjacent bridges contain a double-bond carbon linkage to the ring.

Table 1

KY	R_1	R_2	R3	R_4
KY-1	OH	SO_3Na	H	$>CH_2$
KY-3	OH	SO_2NH_2	H	$>CH_2$
KY-42	OH	SO_3Na	H	$>CHCO_2H$
KY-48	OH	SO_3Na	H	$>CHCHOHCH_2OH$
KY-85	OH	SO_3Na	OH	$>CHC_6H_6$
KY-97	OH	SO_3Na	H	$>CH_2CH=CH_2$
KY-110	OH	SO_3Na	H	$>CHC(O)CH_3$
KY-121	OH	$SO_2C_6H_3(OH)_2$	H	$>CH_2$
KY-123	OH	SO_2Na	H	$>CH_2$
KY-143	OH	SO_3Na	OH	$>CH_2$
KY-147	OH	SO_2NHCH_3	H	$>CH_2$
KY-148	OH	SO_2NHEt	H	$>CH_2$
KY-151	OCH_3	SO_3Na	H	$>CH_2$
KY-158	OH	SO_2CH_3	H	$>CH_2$
KY-171	OH	SH	H	$>CH_2$
KY-175	OH	SO_3CH_3	H	$>CH_2$
KY-176	OH	$SO_2NHC_6H_6$	H	$>CH_2$
KY-193	OH	SO_3Na	Br	$>CHBrCH_2Br$
KY-194	OH	SO_3Na	Br	$>CH_2$
KY-270	$OCOCH_3$	SO_3Na	H	$>CH_2$
KY-272	$OCOCH_3$	SO_3Na	H	$>CHCO_2H$
KY-276	OCOEt	SO_3Na	H	$>CH_2$
KY-277	COEtCl	SO_3Na	H	$>CH_2$
KY-280	OCH_3	SO_3Na	H	$>CH_2$
KY-281	$OCOC_3H_7$	SO_3Na	H	$>CH_2$
KY-284	OCH_3	SO_3Na	H	$>CHCO_2H$
KY-285	$OCOCH_3$	SO_3Na	H	$>CH_2$
KY-288	OCOPr	SO_3Na	H	$>CH_2$
KY-289	$OCOC_4H_9$	SO_3NH_4	H	$>CH_2$
KY-290	OCOBu	SO_3Na	H	$>H_2$
KY-291	$OCOC_5H_{11}$	SO_3NH_4	H	$>CH_2$
KY-293	$OCOCH=CHCH_3$	SO_3NH_4	H	$>CH_2$
KY-294	$OCO(CH_2)_6CO_2H$	SO_3NH_4	H	$>CH_2$
KY-307	$O(CH_2)_5CO_2H$	SO_3NH_4	H	$>CH_2$
KY-346	OH	SO_3Na	H	$-CH_2N(CH_3)CH_2-$
KY-352	OH	$SO_3NHC_6H_{11}O_5$	H	$>CH_2$
KY-357	OH	$SO_2NHCH_2CO_2Na$	H	$>CH_2$
KY-359	OH	SO_2NHOH	H	$>CH_2$
KY-395	OCH_3	SO_3Na	H	$-CH_2N(CH_3)CH_2-$
KY-397	OCH_3	SO_2NH_2	H	$>CH_2$
KY-398	OCH_3	$SO_2NHCH_2CO_2H$	H	$>CH_2$
KY-399	OCH_3	$SO_2NHCH_2CO_2H$	H	$-CH_2N(CH_3)CH_2-$
Y-20	OH	SO_3Na	H	$-CH_2C_4H_2OCH_2-$
Y-34	OH	SO_3Na	H	$-CH_2C_6H_4CH_2-$
Y-66	OH	SO_3Na	H	$>CHCO_2H$
KYY-19	OH	$SO_2NHCH(CH_2)_2(CO_2H)_2$	H	$>CH_2$

Figures 3A and 3B illustrate two preferred synthetic methods for preparing macrocyclic chromotropic acid compounds. The method illustrated in Figure 3A involves cyclization of a chromotropic acid derivative (including chromotropic acid itself) with an aldehyde (RCHO) to form a macrocyclic compound, such as the tetramer shown Figure 2, in which the chromotropic acid subunits are linked by R-substituted methylene groups, i.e., in which R_4 is >CHR (including ≥CR). This synthetic scheme provides a convenient method for constructing macrocyclic compounds having a variety of different bridge-methylene R groups, by carrying out the cyclization reaction in the presence of an aldehyde of the form RCHO.

For example, to construct a macrocyclic compound with a $>CH_2$ bridge, such as the KY-1 compound (IV), chromotropic acid (III) is reacted with formaldehyde. Typical reaction conditions are given in Example 1A for the synthesis of KY-1. Similarly, KY-42 is prepared by cyclization with glyoxylic acid (Example 1C); KY-48, in the presence of glyceraldehyde; KY-85, in the presence of benzaldehyde; KY-97, in the presence of acrolein; and KY-110, in the presence of pyruvic aldehyde. It will be appreciated that a variety of other RCHO aldehydes having small alkyl, alkenyl, acid and other hydrocarbon R groups would be suitable. Further, the R bridge group may be further modified after the cyclization reaction. For example, KY-193 may be prepared by bromination of the KY-97 compound.

In the method illustrated in Figure 3B, cyclization of the chromotropic acid derivatives (III) is carried out by reaction with hexamethylenetetramine, to form a 3-atom chain bridge of the type $-CH_2N(CH_3)CH_2-$ (V). The cyclization reaction for the synthesis of KY-346 is given in Example 1J. The $-CH_2N(CH_3)CH_2-$ bridge may be modified, after the cyclization reaction, to form a variety of N-

substituted bridges of the $-CH_2N(R')CH_2-$, where R' is one of a variety of small carbon-containing groups, according to known synthetic methods. Some of the bridges in the partially oxidized structure will have the form $=CHN(R')CH_2-$.

As noted above, the Figure-4A compound (VI) is representative of macrocyclic naphthalene having a cyclic bridge, in this case a phenyl bridge. The compound is formed by reacting chromotropic acid, in the presence of hydrochloric acid with 1,2-benzenedimethanol in acetic acid, as detailed in Example 3. Similar methods can be employed to link chromotropic acid subunits by other cyclic bridges, such as furan, pyrrole, thiophene, and the like. Figures 4A and 4B show the non-oxidized (VI) and partially oxidized (VIII) forms of the compound.

For synthesis of macrocyclic compounds with selected R_1, R_2, and R_3 substituents, two general approaches are available. In one approach, the chromotropic acid derivative is modified after cyclization so that the cyclized product will either contain the selected R_1, R_2, and R_3 substituent, or contain a substituent which can be readily modified to the selected substituent. This approach is illustrated by the synthesis of KY-3, which has an SO_2NH_2 R_2 substituent, as detailed in Example 1B. Here cyclized chromotropic acid (VIII) is reacted first with chlorosulfonic acid, to form the corresponding $R_2 = SO_2Cl$ derivative (IX, Figure 5). The macrocyclic compound is then reacted with ammonia water to form the desired $R_2 = SO_2NH_2$ derivative (X, Figure 5), as described in Example 1B.

A similar strategy was employed for the synthesis of KY-357 ($R_2 = SO_2NHCH_2CO_2H$) by final subunit reaction with glycine (XI, Figure 5), at basic pH.

Figure 6 illustrates the conversion of sulfonyl groups of cyclized chromotropic acid to sulfinyl (XII) and alkyl

sulfone or methyl sulfinyl ester (XIV). The first stage of the reaction involves the formation of the corresponding sulfonyl chloride derivative (IX), as outlined above. This compound is then treated with sodium sulfite, to form the corresponding sulfinyl salt (XII). Reaction with dimethyl sulfate in the presence of sodium bicarbonate produces the corresponding methyl sulfone (XIV, KY-158, n=4).

Similarly, macrocyclic compounds with a variety of R_1 substituents may be prepared by modification of chromotropic acid after cyclization. In synthesizing KY-151, for example, ($R_1 = OCH_3$) cyclized chromotropic acid is reacted with dimethylsulfate under basic conditions, as detailed in Example 1F, to form the methylether of cyclized chromotropic acid. Similarly, in preparing KY-307 ($R_1 = O(CH_2)_5CO_2H$), cyclized chromotropic acid is first converted to the diether of hexanoic acid by initial reaction of cyclized chromotropic acid with 6-bromohexanoic acid under basic reaction conditions.

As further examples, in preparing compounds such as KY-272 and KY-294, in which R_1 has the form OCOR, the macrocyclic compound formed by cyclization of chromotropic acid is reacted with an acid chloride of the form RCOCl, under basic conditions, as detailed in Example 1I for the synthesis of KY-270.

In a second general approach, the selected substituent is formed on the subunit naphthalene rings by derivatization of the naphthalene subunit, with subsequent subunit cyclization to form the desired macrocycle. For the synthesis of KY-175 ($R_2 = SO_3CH_3$), chromotropic acid is reacted with thionylchloride, as above, to produce the corresponding $R_2 = SO_2Cl$ substituents. Further reaction with $NaOCH_3$ and cyclization leads to the desired R_2 substituent. Reaction details are given in Example 1H.

Among other examples of this approach are KY-123 (Example 1G) and KY-147 (Example 1E).

It will be appreciated that the synthetic method for forming selected-substituent macrocyclic compounds may include both prior derivatization of chromotropic acid and subsequent derivatization of the subunits after cyclization. For example, in forming KY-397 (R_1 = OCH_3, R_2 = SO_2NH_2), chromotropic acid subunits are first reacted at the R_1 positions, to form the methyl ether derivative as described above. After cyclization with formaldehyde, the compound is further derivatized at the R_2 position, also as described above, to convert the SO_3Na group to the desired SO_2NH_2 substituent.

The KY compounds described above can be converted readily to a variety of sulfonic acid or sulfonate salts, by reaction in acid or in the presence of a suitable salt, according to well known methods. Thus, for example, several of the KY compounds shown in Table 1 are ammonium salts formed by cation exchange of protons in the presence of an ammonium salt, such as ammonium chloride. In addition, exposure of the macrocyclic compound to a variety of metal cations, such as the cations of Ca, Ba, Pt, Cu, Bi, Ge, Zn, La, Nd, Ni, Hf, or Pb, may produce both a metal salt and a metal chelate of the macrocyclic compound in which the metal is chelated at interior polar pocket in the compound.

The physical properties of several macrocyclic compounds prepared in accordance with the invention have been studied by absorption and mass spectrometry and by nuclear resonance spectroscopy (NMR), as detailed in Examples 1A, 1B, 1C, and 1J. These compounds include tetrameric macrocyclic compounds, such as indicated in Figure 2, or mixtures with predominantly tetrameric forms.

B. Macrocyclic Compounds with Substituted Phenol Subunits

Figure 7 shows the general structural formula of a macrocyclic compound composed of substituted phenol subunits, for use in the present invention. One exemplary compound of this type is shown in Figure 8, which is a tetramer of phenol para-sulfonic acid subunits linked by methylene bridges (XV). As seen, the methylene bridges and the "interior" ring atoms (ring positions 2, 1, and 6) form a continuous chain having R_1 = OH groups attached at the 1 ring positions. The non-chain atoms (ring positions 3-5 on each substituent) have R_2 = sulfonic acid substituents on the 4 ring atoms.

Figure 9A illustrates a general method for forming macrocyclic compounds of this type. The macrocyclic precursor shown at the left (XVI) is a class of compounds known generally as tert-butyl calix(n)arenes, where n is the number of phenolic subunits (with para-position t-butyl substituents) in the macrocycle, and the bridge connections are methylene groups. t-butyl calixarenes with 4, 6, and 8, subunits are commercially available.

In the sulfonation reaction shown in Figure 9A, a t-butyl calixarene with a selected subunit number is treated with concentrated sulfuric acid, typically for about 4 to 5 hours at 75-85°C to effect substantially complete displacement of the 4-position t-butyl group by a sulfonic acid group. Details of the sulfonation reaction are given in Example 2A. The method has been used to produce the n=4 macrocycle compound shown in Figure 8, and related macrocycles with 6 and 8 phenol subunits.

A similar method is used for preparing a sulfonated calixarene with partially oxidized 1-position OH groups, as shown at 9B. Here the t-butyl calixarene starting material is treated with conc. sulfuric acid at a

temperature above 100°C, preferably between 150-170°C. The reaction is effective to sulfonate the subunit rings and to partially oxidize the interior OH groups. As indicated in Figure 9B, partial oxidation can lead to a conjugated macrocyclic structure (XVIII) in which bridge contributes delocalized electrons. This conjugated structure is colored, and the development of a colored product can be used to monitor the course of the oxidation reaction. Details of the reaction are given in Example 2B.

It will be appreciated that the desired macrocycle can also be formed directly by reacting para-sulfonic acid phenol (or precursors thereof) under suitable bridging conditions, such as described above for producing naphthalene-subunit macrocycles. This is illustrated by the reaction shown in Figure 12, for production of a macrocyle having carboxylic acid-containing bridge groups. In this method, phenol para-sulfonic acid is reacted with glyoxylic acid, under conditions similar to those described in Example 2C, to form the cyclized structure shown (XXII).

The macrocyclic compounds formed as above can be modified, according to general procedures outlined in Section IIA above, to achieve selected R_1 groups, modified sulfonyl groups, and/or addition of R_2 groups. The range of R_1 and R_2 substitutents is substantially the same as that discussed above. Figures 10, 11, and 13 illustrate various reaction methods for modifying the R_1 group of an already formed macrocycle. In Figure 10, the sulfonated structure shown in Figure 8 is treated with acetic anhydride, to form an O-acetyl R_1 group. Details of the reaction are given in Example 2C. Since this structure would be expected to undergo hydrolysis in the presence of serum esterases, differences in the activity of the ester compound and the free OH compound would be

expected to occur after intravenous (IV) administration. Example 2G describes a similar reaction scheme for forming a toluene sulfonic acid ester at the R_1 position.

Figure 11 illustrates a general method for forming sulfonamides, such as glycylsulfonamide (XXI) of the Figure 8 compound. Analogous to the reactions described with respect to Figure 5, the sulfonated phenyl macrocyclic compound (XVII) is treated with chlorosulfonic acid, to form the corresponding sulfonyl chloride analog (XX). Further reaction with a selected amine, in this case glycine, gives the desired sulfonamide. Reaction details are given in Example 2D for the synthesis of the $R_2 = SO_2NH_2$ compound and in Example 2E, for the synthesis of the glycyl sulfonamide compound.

Figure 13 depicts a general synthetic method for a net substitution of $R_1 = OH$ by R_1 = carbon moieties. In Example 2H, the reactions detail a process from which a substrate ($R_1 = OH$, R_2 = tert-butyl, $R_4 = CH_2$, $n = 4$) affords an intermediate ($R_1 = CN$, R_3 = tert-butyl, $R_4 = CH_2$, $n = 4$). Further modification then provides the product ($R_1 = CO_2H$, $R_3 = SO_3H$, $R_4 = CH_2$, $n = 4$).

It will be appreciated that substituent modifications at the R_1 site can be selectively carried out at OH sites in the partially oxidized macrocycle, such as the structure shown at Figure 9B. That is, reactions which are specific for ring OH groups will leave the =O group intact, thus providing a mixed R_1 group containing =O groups.

The R_4 bridge linking the chromotropic acid derivative subunits is preferably of the form >CHR or $\geq$ CR, where R is H or a small carbon-containing group, such as lower alkyl, alkenyl, ketone, or carboxylic acid group, or aryl group, as noted above, or of the form $-CH_2NR'CH_2-$, where R′ is similarly H or a small carbon containing group,

such as a lower alkyl group. Alternatively, the bridges in the macrocycle may be ring structures, including aryl ring structures, analogous to the dimeric macrocycle shown in Figure 4.

Also as above, the number of subunits may vary from 4 (e.g., Figure-4 structure) to 8, with macrocycles containing 4, 6 and 8 subunits being preferred. In the reaction schemes described below, the macrocycle formed may include mixtures of compounds with different subunit numbers (n)
values, e.g., a dominant n=4 structure (4 subunits) plus additional structures containing 5-8 subunits.

Representative macrocyclic compounds which have been synthesized and tested for anti-viral activity are identified by their R_1, R_2, and R_4 substituents in Table 2 below. The KY and Y number in the lefthand column in the table refers to the analog designation of the corresponding compound, as in Table 1. Compounds which are partially oxidized at the R_1 position, and have which may have both saturated and unsaturated bridge methylene carbon groups are indicated as in Table 1.

Table 2

Compound	R_1	R_3	R_4	n
Y-1	OH	SO_3	$-CH_2-$	8
KY-226	O/OH	SO_3	$-CH_2/=CH-$	8
Y-49	OH	SO_3	$-CH_2-$	4
KY-225	O/OH	SO_3	$-CH_2/=CH-$	4
Y-77	OH	SO_3	$-CH_2$	6
Y-48	O/OH	SO_3	$-CH_2/=CH-$	6
KY-268	O/OH	SO_3	$-CH_2/=CH-$	3
KY-269	O/CO_2CH_3	SO_3	$-CH_2/=CH-$	4
KY-271	O/CO_2CH_3	SO3	$-CH_2/=CH-$	3
Y-78	O/OH	SO_2NH_2	$-CH_2-$	8
Y-100	O/OH	SO_2OCH_3	$-CH_2-$	8

The compounds shown in Table 2, and R-group combinations thereof, described above can be converted readily to a variety of sulfonic acid or sulfonate salts, by reaction in acid or in the presence of a suitable salt, according to well known methods, as described above.

III. Anticoagulant properties of macrocyclic compounds

This section describes the ability of compounds useful in the invention to inhibit coagulation of blood, as shown in one or more standard blood coagulation assays. Assays which are used in assessing anticoagulant activity and, to some degree, mechanism of anticoagulant activity, include, but are not limited to the activated partial thromboplastin time (APTT) assay, the prothrombin time (PT) assay, the thrombin time (TT) assay, the fibrinogen assay, the reptilase (atroxin time, AT) assay, and the plasma clotting (recalcification) time assay. Such assays and specific methods for carrying them out are

known generally in the art and are described by Brown (1988).

The blood used to test compounds in such assays may be from a variety of vertebrate sources, although mammalian, and particularly human sources are preferred. In carrying out such assays, venous blood samples are obtained using clean venipuncture procedures, in order to prevent contamination of the sample by exogenous cells. Blood samples employed in the screening compounds useful in the method of the invention may be collected in any of a number of standard collection tubes holding a calcium binding or chelating agent. Plastic tubes are preferred; however, glass-walled VACUTAINER™ tubes containing sodium citrate as a calcium binding agent are adequate in practicing most experiments supporting the invention. Freshly drawn samples are stored at ice temperature for up to 2-4 hours prior to further processing, and are checked for the presence of clots or hemolysis; any tubes containing clots are discarded. Plasma is obtained from the samples, using centrifugation procedures described in Example 4A. Plasma samples showing evidence of hemolysis are discarded, since hemolysis is known to shorten clotting time. Ideally, plasma samples are stored on ice and tested within 8 hours of collection. Alternatively, the samples may be frozen at -20° for testing within 1 week of collection. General methods used in collecting and processing blood samples for experiments in support of the invention are found in Example 4A.

Tests for anticoagulant activity may be carried out <u>in vitro</u>, wherein compound is added to an isolated plasma or blood sample, and effects on clotting time are measured. Anticoagulant activity may also be measured following administration of a compound to a whole animal. Such <u>in vivo</u> assessment of compound effects indicates the degree to which a drug is absorbed, distributed or

biotransformed in the whole animal, and gives a measure of bioavailability.

A. Testing of Macrocyclic Compounds in Anticoagulant Assays

1. Plasma Clotting (Recalcification) Time

Plasma clotting or recalcification time measures the integrity of intrinsic coagulation system. A deficiency or inhibition of any of the factors of the intrinsic system results in prolongation of plasma clotting time. Both heparin and coumarin anticoagulants prolong plasma clotting time. In this assay, as described in Example 4D, plasma is mixed with calcium chloride at 37°, mixed and observed for clot formation.

Effects of compounds on clotting time in vitro

Results of studies in which various macrocyclic compounds were tested at three concentrations for effects on plasma clotting time in vitro, as described in Example 5 are shown in Table 3. Phenylic macrocyclic compounds KY225 and Y-47 exhibited the highest anti-coagulant activity in this assay. Concentrations of 12.5 μg of each of these compounds produced anticoagulant activity equivalent to 7.54 and 5.68 μg of heparin, respectively. Phenylic derivatives Y-48, Y-77, Y-78, Y-100 and Y-1 and napthylic derivatives Y-20, KY-42, KY-1, KY-357 and KYY-19 were approximately equipotent in the assay, exhibiting activities about 1/10-1/20 that of heparin on a mass basis.

Table 3

COMPOUND	CONCENTRATION (μg/ml)		
Phenyl derivatives	12.5	25	50
KY-384	0.4	1.38	3.31
Y-48	1.2	2.94	5.29
KY-225	7.54	>>[a]	>>
Y-47	5.68	15.27	>>
KY-226	-	-	1.10
Y-1	1.10	1.68	3.68
Y-77	1.10	1.27	3.22
Y-49	-	-	0.46
Y-78	1.40	2.45	4.03
Y-100	1.40	1.93	2.19
Napthyl derivatives	12.5	25	50
KY-3	-[b]	1.5[c]	3.68
KY-42	1.1	2.94	4.16
Y-20	1.1	2.94	4.88
KY-332	-	-	1.66
KY-274	0.46	0.92	2.67
KY-1	1.10	1.38	3.96
Y-36	0.4	1.30	3.50
KY-357	1.40	2.28	3.5
KYY-19	1.40	2.28	4.03

[a] Anticoagulation activity is to high to be measured in the assay.
[b] Not detectable in anti-coagulant activity.
[c] Values are expressed as heparin microgram equivalents; i.e., 25 μg KY-3 produces anticoagulant activity equivalent to 1.5 μg heparin.

Effects of Y-1 on clotting in vivo

The effects of Y-1 on clotting time following oral administration were studied in mice, as described in Example 13. Table 4 shows the results of a study in which two doses of 500 or 625 mg/kg each of Y-1 were administered to female Swiss-Webster mice at 30 minute intervals by gastric gavage. Blood samples were collected 2.5 hours following the initial dosing. Blood plasma was assayed for plasma clotting time. Compared to control (PBS-treated) animals, Y-1-treated animals showed increased clotting time, at both doses tested. For purposes of comparison, Y-1 (12 or 20 μg/ml) was also added directly to plasma samples from control animals, and plasma clotting times obtained were within the range of the times reported after oral administration of the compound.

Also shown in Table 4 is protamine reversal of the effects of Y-1 on plasma clotting time. This will be described in further detail in Part 4, below.

Table 4

Effect of Oral Y-1 on Recalcification Clotting Time and Reversal by Protamine Sulfate

Y-1 Treatment (mg/kg)	Nutritional Status[1]	Clotting Time	
		No Addition	+ Protamine Sulfate (10.4 μg/ml)
0[2]	N	2.2	n.d.
2 × 500	F	4.5	2.4
2 × 625	N	3.1	2.3
2 × 625	F	3.4	2.4

1 N, not fasted; F, fasted 24 hours prior to test

2 Addition of Y-1 directly to control plasma gave clotting times of 3.2 min (12 μg/ml Y-1) and 4.1 min (20 μg/ml Y-1).

2. Prothrombin Time (PT) Assay

Prothrombin time assesses the patency of the extrinsic coagulation pathway, and measures the presence of factors II, V, VII, and X. This assay also serves as an indicator of levels of fibrinogen less than about 80 mg/dL. Prothrombin time is therefore useful in assessing therapy by coumarin anticoagulants, which inhibit production of factors II, VII, IX, and X. The presence of relatively high concentrations of heparin in blood samples also prolongs prothrombin time measurements.

Methods used in determining PT can be found in Example 4B. Briefly, the assay involves the addition of a tissue factor, such as thromboplastin-calcium reagent (Dade® Thromboplastin•C, Becton Dickinson) to a plasma sample. The duration of time from the time of addition until visible clot formation is observed is the PT.

a. Effects of In vitro Administation of Macrocyclic Compounds on Prothrombin Time

Macrocyclic compounds KY-1, Y-1 and Y-49 were tested in a PT assay using human blood, as described in Example 6. Human plasma samples containing varying amounts of test compound (0-250 μg/ml, final concentrations) were tested clotting time subsequent to mixing with thromboplastin-calcium reagent.

Of the several macrocyclic compounds tested in this assay, KY-1 exhibited highest activity (Figure 15), exhibiting prolongation of prothrombin time at concentrations as low as 30 μg/ml. Y-1 showed intermediate activity. Y-49 was inactive at concentrations as high as 900 ug/ml (not shown in graph; but in raw data).

b. In vivo administration of macrocyclic compounds

Macrocyclic compounds of the invention were given intravenously to rats at doses of 5 and 10 mg/kg (2 rats/dose). Subsequently (5 hours following administration) venous blood samples were tested for PT, APTT, and fibrinogen. Table 5 shows the percent change in PT observed, as compared to the PT of blood plasma from saline-treated controls. Moderate increases in PT were observed for several of the compounds tested, most notably KY-225 and KY-226, partially oxidized macrocyclic compounds having 4 and 8 phenyl subunits, respectively.

Table 5

Effect of i.v. Administration of Macrycyclic Compounds on PT, APTT and Fibrinogen

Phenyl Derivatives	Dose (mg/kg)	PT	APTT	Fibrinogen
KY225	5 10	21 35	81 >500	N[1] -40
KY226	5 10	25 30	93 >500	N -26
Y1	5 10	12 28	(67)[2] 66	7 N
Y48	5 10	12 11	23 138	-14 -17
Y49	5 10	N 7	6 27	N N
Y47 (SA)	5 10	26 16	(54) 90	N -17
KY-384 (SA)	5 10	N 6	70 151	N -12
Napthyl Derivatives	**Dose (mg/kg)**	**PT**	**APTT**	**Fibrinogen**
KY-1	5 10	20 88	>500 >500	-21 -59

Y20	5 10	N 10	(82)[3] 29	N N
KY3	5 10	N 10	N 38	N 13
KY42	5 10	11 19	6 61	N N

[1] N, no effect
[2](3 and 131%)
[3](21 and 142%)

In a separate series of studies, Y-1 was administered at oral (p.o.) doses of 300 and 450 mg/kg to rats. Blood samples were taken and PT determinations made at times from 0.5 to 24 hours following administration, as described in Example 14. Results of these experiments are shown in Table 6, wherein significant increase (18%) in PT was observed 4 hour post-administration of 450 mg/kg Y-1 by gastric gavage. Reproducibility of this effect was tested by administering additional compound to some of the animals at 23 hours and testing PT at 24 hours. Once again, a significant (19%) prolongation of PT was observed. APTT prolongation was also observed at both doses of Y-1, as described below.

Table 6

Time Course of Effect of Oral Y-1 on Plasma PT and APTT

I. 300 mg/kg p.o.

Time after Administration (h)	PT[1]	APTT[2]
0[1]	16.2 ± 0.2	13.0 ± 0.1
1	15.7 ± 0.2	20.3 ± 0.96
2	15.8 ± 0.1	19.7 ± 0.6
4	16.0 ± 0.3	18.5 ± 1.3

Time after Administration (h)	PT[1]	APTT[2]
8	16.6 ± 0.1	19.5 ± 0.8
12	16.7 ± 0.2	15.4 ± 0.5
24	16.6 ± 0.2	14.3 ± 0.2

II. 450 mg/kg p.o.

Time after Administration (h)	PT[1]	APTT[2]
0[1]	14.8 ± 0.32	18.9 ± 1.3
0.5	15.4 ± 0.45	32.4 ± 1.8
4	17.5 ± 0.56	53.2 ± 9.1
8	15.1 ± 0.2	24.7 ± 0.7
16	15.5 ± 0.1	23.2 ± 1.5
24	15.0 ± 0.2	21.5 ± 0.5
24, repeat @ 23[3]	17.6 ± 0.14	48.4 ± 5

1 Zero time control animals given saline

2 PT and APTT in seconds, mean of 4 animals ± SE.

3 Animals previously treated with 450 mg/kg p.o. were given an additional oral dose of 225 mg/kg at 23 h.

3. Activated Partial Thromboplastin Time (APTT) Assay

The APTT assay is employed as a measure of the integrity of the intrinsic blood coagulation pathway, described above. It measures the presence of all coagulation factors in the intrinsic system except platelets and factor XIII, and is commonly used to monitor heparin therapy, since heparin binds to several of the factors of the intrinsic pathway (XIa, IXa, Xa, thrombin).

Detailed methods used in carrying out this assay can be found in Example 4C. Briefly, the plasma sample is mixed with activated thromboplastin, such as Actin® Activated Cephaloplastin Reagent (Becton Dickinson). The tube containing the mixture is placed in a 37° water bath for 3 minutes, prior to addition of calcium chloride. The sample is then observed for fibrin web formation.

a. Effects of macrocyclic compounds on APTT, in vitro

Platelet poor plasma (human) was used to test the effects of KY-1, Y-1 and Y-49 on APTT, as described in Example 7. Figure 16 shows the results of these experiments, in which varying concentrations of each compound were tested, to yield concentration-effect plots. Of the three compounds tested, KY-1 produced the highest activity in this assay, and Y-1 exhibited less activity. Y-49 was inactive at the highest concentration tested.

In separate experiments, compound KY-1 was added to a human blood sample at a concentration of 50 μg/ml, and tested in a battery of standard clinical tests, one of which was APTT. A significant prolongation of APTT was observed, as shown in Table 8, part 9 of this section.

b. Effects of in vivo administation of macrocyclic compounds on APTT

Results from experiments in which rats were given macrocyclic polysulfonated compounds intravenously are shown in Table 5, above. Percent change in APTTs was determined, in comparison to untreated animals. With few exceptions, compounds described in the method of the invention exhibited a dose-dependent prolongation of APTT. Specifically, partially oxidized phenylic derivatives KY-225 and KY-226 exhibited the highest activities in this assay, while Y-48 and Y-42 followed,

in descending order of activity. Compounds Y-49, Y-20 and KY-3 showed slight activity.

The observation that macrocyclic polysulfonated compound treatment of whole animals resulted in prolongation of APTT was further examined, using rats as test animals. In this case, illustrated in Figure 17, compound Y-1, was given intravenously to animals at a doses of 2.5 and 5 mg/kg, and blood samples were drawn from four different animals at various times following administration. APTT induced an immediate prolongation of APTT to approximately 300% of normal or higher, with the anti-coagulant effect persisting up to 4-6 hours after the 2.5 mg/kg dose and remaining approximately 20% abve normal at 12 hours after the 5 mg/kg dose. Data are expressed as percent of control values for each set of animals to normalize values obtained in different experiments.

Figure 18 illustrates the APTT dose-response relationship of Y-1 by plotting the 30-minute values of separate experiments in which 2.5, 5 and 25 mg/kg of Y-1 were administered intravenously to rats. High doses of Y-1 exerted profound effects on APTT, and the linearity of the dose-response curve demonstrates a high degree of predictability for the anticoagulant effect of Y-1.

The effects of oral administration of Y-1 were also tested, in assays described above for PT. As shown above in Table 6, a prolongation of APTT was observed at both 300 and 450 mg/kg Y-1. At the 450 mg/kg dose, this effect peaked at about 4 hours post-administration but was still apparent 16 hours post-administration. These studies confirmed that macrocyclic compounds of the invention are active when administered orally, and that their effects are relatively long-lasting.

4. Reversal by Protamine Sulfate of Anticoagulant Effects of Macrocyclic Compounds

Blood or plasma samples treated with macrocyclic compounds of the invention were treated with protamines, to determine their effect on compound-induced anticoagulant activity, as assessed by several of the test assays used in experiments in support of the present invention. Murine plasma was tested for clotting time subsequent to oral administration of compound Y-1, as described in Example 13, and shown above in Table 4. In these studies, protamine sulfate was added to plasma samples from Y-1 treated animals at a concentration of 10.4 μg/ml. Addition of protamine sulfate to the samples resulted in reversal of the Y-1-induced prolongation of clotting times.

The efficacy of protamine sulfate in reversing the effects of Y-1 was further tested in rats, using the protocol described in Example 14, except that animals were given 25 mg/kg Y-1 intravenously. Twenty-six to twenty-eight minutes later, protamine sulfate was administered, also intravenously, at a dose of 25 mg/kg. Blood samples were collected 30 minutes after initial drug injection. Results of these studies are shown in Table 7. Treatment of rats with Y-1 resulted in elevation of PT and APTT to 161% and 831% of PBS control values, respectively. Protamine sulfate treatment resulted in a total reversal of the effects of Y-1 administration on PT and an almost total reversal of the effects on APTT.

Table 7

Effect of Intravenous Y-1 and Protamine Sulfate on PT and APTT *in vivo*[1]			
Treatment	N	PT (sec)	APTT (sec)
Saline	4	14.8 ± 0.3	18.9 ± 1.3
Y-1 (25 mg/kg)	4	23.9 ± 0.7	157.1 ± 11.5
Y-1 (25 mg/kg) +protamine sulfate (25 mg/kg)	4	13.9 ± 0.1	27.6 ± 2.8

[1] Values expressed as mean ± std. error

4. Fibrinogen Assay

Fibrinogen is the polymeric precursor of fibrin monomers, which spontaneously polymerize to initiate clot formation. In the coagulation cascade, as illustrated in Figure 14, fibrinogen is converted to fibrin by the proteolytic action of thrombin. Fibrinogen content of blood may be affected by a number of insults. Lack of fibrinogen reduces clot formation. The presence of relatively high concentrations of heparin in samples can result in an artificially low value for fibrinogen content as determined by the thrombin time assay (see following section), due to inhibition by heparin of endogenous and added thrombin.

Fibrinogen content of blood can be measured by adding an excess of thrombin to a dilute plasma sample and recording clotting time, as described in Example 4E. Fibrinogen contents of plasma samples taken from rats previously given intravenous doses of various macrocyclic compounds are shown in Table 5, above. At higher doses, it is apparent that KY225, KY226, and, to a lesser degree, Y-48 treatment resulted in a decrease in fibrinogen content of the blood. In addition, in a study

in which KY-1 was added directly to human blood at a concentration of 75 μg/ml prior to assaying the blood plasma in a standard battery of clinical tests, a profound decrease in fibrinogen content was observed (shown in Table 8, below).

5. Thrombin Time

Thrombin time is another measure of the conversion of fibrinogen to fibrin, catalyzed in the blood by the enzyme thrombin. Prolonged thrombin times can be caused by a number of factors, including low fibrinogen levels, heparin, and other thrombin inhibitors such as fibrin degradation products.

The assay is carried out by adding a stock quantity of purified thrombin to platelet poor plasma samples, as described in Example 4G and recording the amount of time required for clot formation in the plasma.

Results of studies in which the compounds KY-1, Y-1 and Y-49 were tested for effects on TT, as described in Example 8, are shown in Figure 19, where it is seen that the presence of KY-1 in the plasma sample markedly increased thrombin time, whereas Y-49 showed no activity at the concentrations tested.

6. Reptilase Assay (Atroxin Time)

Reptilase, an enzyme isolated from snake (Bothrops atrox) venom, which converts fibrinogen to fibrin, is not affected by heparin. It is therefore useful in testing for fibrinogen content of the blood of patients receiving heparin therapy. Blood from patients receiving a fibrinolytic agent, such as streptokinase, exhibits prolonged atroxin and thrombin times. General procedures for carrying out reptilase assays are described in Example 4F.

Figure 20 shows the effects of increasing concentrations of KY-1, Y-1, and Y48 on atroxin time of human plasma in vitro. KY-1 prolongation of atroxin time only occurred at concentrations approaching 150 μg/ml, a concentration much higher than that (25 μg/ml) required to significantly prolong thrombin time, as illustrated in Figure 19. These results are similar to those observed with heparin, wherein high concentrations can be shown to prolong reptilase time, and indicate that the effect of KY-1 on fibrinogen content, reported in Part 4, above, can be explained at least in part by an indirect, heparin-like effect on the thrombin present in the assay.

7. Platelet Aggregation

Compounds, such as aspirin, which interfere with platelet aggregation result in prolonged bleeding time. Integrity of platelet aggregation in a blood sample can be measured by a characteristic change in optical density of a platelet rich plasma sample in response to platelet aggregation promoting factors, such as ADP or collagen, as described in Example 4H. In studies on collagen activated platelet aggregration, carried out as described in Example 16, KY-1 and Y-49 at 24 μg/ml and 48 μg/ml concentrations had no measureable effect on collagen-induced aggregation (Figures 21, B, C, F, G). Y-1 at 24 μg/ml and 48 μg/ml showed significant inhibition of collagen induced platelet aggregation (Fig. 21 D, E).

8. Plasmin Activity

Compounds KY-1, Y-1 and Y-49 were tested for plasmin chromogenic effects, as described in Example 4I. Figures 22A and 22B show the results of these studies. Both KY-1 and Y-1 exhibited concentration dependent effects on plasmin chromogenic activity. In terms of enzymatic

activity, measured as change in absorbance units per minute, the inhibitory effect of Y-1 at 20, 40, and 80 μg/ml were 15%, 28%, and 31% of control plasmin activity respectively (Figure 22A); for KY-1 at 9 and 19 μg/ml, 35% and 52% of control activity. At higher doses of KY-1 and C8, the inhibition was still modest, KY-1 (64 μg/ml) caused 35% inhibition and Y-1 (233 μg/ml) 34% inhibition. Heparin at an equivalent anticoagulant dose in terms of TT had no inhibitory effect (Figure 22B).

9. Effect of KY-1 on human plasma in Clinical assays

A human blood sample was drawn, and KY-1 was added to a final concentration (75 μg/ml), prior to transport of the sample to a licensed clinical testing lab for a battery of standard clinical assays. Results of these tests are shown in Table 8. It is apparent that at the concentration of compound used, all standard assays registered abnormal coagulation parameters.

Table 8

Effect of KY-1 75 μg/ml) on Coagulation of Human Plasma: Comparison of Clinical Assays

Assay	KY-1 Treated	Normal Range
Prothrombin Time (PT)	26.8 sec	11-13 sec
APPT	>300 sec	24-34 sec
Fibrinogen	<30 mg/dL	160-350 mg/dL
Thrombin Time	>100 sec	13-17 sec
Reptilase Time	48.1 sec	9.6-14.0 sec

9. Summary of Effects of Macrocyclic Compounds on Blood Coagulation

KY-1 and Y-1 serve as prototype napthyl and phenyl macrocyclic compounds in analyzing the effects of these classes of compounds on blood coagulation. Both compounds were observed to prolong overall clotting time, with approximately equivalent potencies (Table 3); as described above, and summarized below, these compounds may have slightly different predominant mechanisms of action.

KY-1 significantly prolonged PT, APTT, TT and AT *in vitro* (Figure 23). However, concentration-effect studies (0.6-180 μg/ml) clearly showed that the KY-1 prolongation of AT only occurred at concentrations approaching 150 μg/ml, similar to a heparin-like effect. To determine if this was entirely due to a heparin-like effect, a further experiment was done in which heparin and KY-1 were tested at concentrations that gave similar prolongation in thrombin times (Figures 24 A-B). KY-1 at 19 μg/ml which gave TT of 38 sec showed marked prolongation of APTT to >300 sec, while heparin at 0.41 μg/ml with TT of 49 sec gave APTT of 81 sec. These data suggest that the KY-1 anticoagulant effect is not entirely due to a heparin-like effect.

Y-1 also prolonged PT, APTT, TT and AT. In contrast to KY-1, the effect of Y-1 on AT was minimal, with no prolongation at 400 μg/ml (Figure 20). Concentration-effect studies showed significant prolongation of both PT and PTT (≈ 2 × baseline) at 20 μg/ml with no significant prolongation in TT at this concentration. TT prolongation was only seen at ≥ 75 μg/ml (Figure 19). These data suggest that the anticoagulant effect of Y-1, at ≈ 20 μg/ml range, is most likely not due to a heparin-like effect. The simultaneous prolongation of PT and aPTT suggests that Y-1 may be directed against the common

pathway. Potential targets include Factor X, Factor V, prothrombin (Factor II) or phospholipids. Additionally, Y-1 exhibited anti-platelet activity.

The different activity profiles of Y-1 and KY-1, which are taken to be representative of the macrocyclic phenyl- and macrocyclic napthyl derivatives respectively, suggest that the compounds may be preferred in different indications requiring anticoagulant therapy. For example, in those cases, such as incertain forms of valvular heart disease, in which concurrent anti-platelet and anti-coagulant therapy is indicated, compounds exhibiting the Y-1 activity will be indicated.

Both Y-1 and KY-1 were shown to be active in vivo, when administered either parenterally or orally. Peak effects after oral administration were dose dependent and were observed between about 0.5 and 4 hours following oral administration of 450 mg/kg Y-1, as assessed by APTT (Table 6). The apparant distribution half-life of this compound, following intravenous administration of 2.5 and 5 mg/kg, as assessed by APTT, is less than an hour, and elimination half-life about 3-4 hours with APTT remaining above control levels for at least 4 hours in a dose-related manner (Figure 17).

Studies using Y-1 demonstrated that, although as stated above, the compound does not appear to have a heparin-like mechanism of action, its effects can be antagonized by protamine sulfate. This finding suggests relatively convenient, approved antidote to accidental overdosage with the compound.

IV. Method of Treatment

In the method of treatment of the invention, an aryl macrocyclic compound of the type described in Section II is administered to the bloodstream of a patient at risk for developing thromboembolism. As described above, the

compounds of the invention appear to have direct effects on a factor or factors present in the coagulation cascade, by which they produce their anticoagulant effects.

The main routes of drug delivery are intravenous and oral, with the preferred route being oral. Other drug-administration methods, such as intra-arterial, subcutaneous, or nasal insufflation, which are effective to deliver the drug into the bloodstream, are also contemplated.

The dosage which is administered is a pharmaceutically effective dose, defined as a dose effective to prolong coagulation time of blood in a patient. As seen above, compound concentrations in the range of 10-100 μg/ml are generally effective to inhibit coagulation, as assessed by plasma recalcification time, APTT or PT, in vitro. Thus, for most indications, an effective dose would be one which produces a concentration of compound in this range in the blood.

One consideration in any anticoagulant therapy regimen, in view of its inherent potential for producing life-threatening hemorrhage, is identifying an antidote to the therapy; that is, a mode of abrupt discontinuation of action of the compound in the event of overdosage of the compound. In the method of treatment of the present invention, an effective antidote, protamine sulfate, has been identified. In the event of overdosage, it is anticipated that an amount of protamine sulfate approximately equal to or less than the amount of compound administered, would be effective to antagonize the effects of the drug, the amount of protamine sulfate dependent on the time after administration of the compound.

A. Treatment by Intravenous Administration

Studies on the pharmacokinetics and efficacy of intravenously administered macrocyclic compounds of the invention are described above. Briefly, aryl macrocyclic compounds produce anti-coagulant effects for 4 hours or longer in a dose-related manner following intravenous administration.

B. Treatment by Oral Administration

Studies in support of the present invention, described above, show that following oral administration (gavage), macrocyclic compounds of the invention produce significant anti-coagulant activity for 4 or more hours, the duration of effect being dependent on the dose administered.

The following examples illustrate methods of preparing tetrameric macrocyclic compounds, in accordance with the invention, and their use in inhibiting blood coagulation by enveloped viruses. The examples are intended to illustrate but not limit the scope of the invention.

Materials

All chemical reagents were obtained from Aldrich Chemical Co., or from other commercial sources.

Example 1

Preparation of Naphthalene Macrocyclic Compounds

A. KY-1 (R_1=OH, R_2=SO_3Na, R_3=H, R_4= $>CH_2$)

To a 41 mM aqueous solution (50 ml) of disodium chromotropic acid, 15 ml of 37% formaldehyde was added, giving a final molar ratio of 5:1 formaldehyde:chromotropic acid. The mixture was reacted with stirring in a stoppered flask at room temperature for 1 week. The resulting dark red solution (70 ml) was filtered under

vacuum, and the filtrate, after being concentrated was precipitated by adding 200 ml of acetonitrile. The precipitated product was collected by filtration and taken to dryness under vacuum. The yield of KY-1 was 95%. The compound was characterized as follows:

Melting point (M.P.)> 300°C;

HPLC in $CH_3CN/MeOH/H_2O/TFA$: 14'48" single broad peak;

(IR/KBr) = 3425 (OH), 1638 (Ar), 1181, 1044 (SO_3) cm^{-1}:

UV (H_2O): 238.0, 358.5 nm

Mol Weight: 1505 (M+1) by mass spectroscopy;

H^1 NMR(CD_3OD), chemical shifts on the δ scale: 5.20 (CH_2, 8.01 (ArH) ppm;

C^{13} NMR (D_2O), chemical shifts on the δ scale: 27.19, 120.18, 121.69, 122-06, 122-67, 133-30, 142.97, 154.42 and 181 ppm. Analysis: $(C_{22}H_{10}O_{16}S_4Na_4)_2$ x 6 H20 or $(C_{22}H_{11}O_{16}S_4Na_4)_2$ x 5 H_2O

Found: C 33.17, H 2.54, Na 11.93

Calculated: C 32.75, H 2.23, Na 11.41; C 33.16, H 2.13, Na 11.56.

B. KY-3 (R_1=OH, R_2=SO_2NH_2, R_3=H, R_4= $-CH_2-$)

KY-1 (2mM) was treated with 5 ml chlorosulfonic acid and the mixture was stirred at 50°C for one-half hour. The resultant mixture was added to 20 g of crushed ice to precipitate the crude chloride product, which was collected by filtration and then washed with ether.

The crude chloride product was dissolved in 100 ml of 25% ammonium water solution and allowed to react for 2 hours at room temperature. The mixture was concentrated in vacuo and the remaining oil was dissolved in a small amount of water and filtered. The product was precipitated by adding acetonitrile to the filtrate and collected by filtration and washing with acetonitrile. The compound was characterized as follows:

Melting point (M.P.)> 300°C;
Mass spec: 1452 ($M-7NH_2$);.
HPLC in $CH_3CN/MeOH/H_2O/TFA$: 11'46" single peak;
(IR/KBr) = 3430 (OH), 3187, 1686 (NH_2), 1637 (Ar), 1211, 1110, 1044 (SO_3) cm^{-1};
UV (H_2O): 246 nm;
H^1 NMR(D_2O), chemical shifts on the δ scale: 5.15 (CH_2), 7.5-8.2 (ArH) ppm;
Analysis: $(C_{44}H_{40}O_{26}S_{10}N_{12}Na_4)-16H_2O$
Found: C 28.62, H 3.93, N 8.82, S 17.17, Na 5.44;
Calculated: C 28.51, H 3.89, N 9.07, S 17.28, Na 4.97;

C. KY-42 (R_1=OH, R_2=SO_3Na, R_3=H, R_4= >CHCOOH)

Chromotropic acid, disodium (10mM) in 50 ml water was mixed with glyoxylic acid (10.0 mM, in 5 ml water) and 10 ml of 37% hydrogen chloride at room temperature. The mixture was boiled for 8 hours and the color of the solution turned to dark red. The resultant solution was added to 50 ml of water and filtered. The filtrate was concentrated and ethanol was added to precipitate the product of KY-42. The yield was 87%. The compound was characterized as follows:
Melting point (M.P.)> 300°C;
Mass spec: 1623 ($M-3H_2O$).
HPLC in $CH_3CN/MeOH/H_2O/TFA$: 10'36" single peak;
(IR/KBr) = 3452 (OH), 1801, 1719 (Co), 1638 (Ar), 1206, 1050 (SO_3) cm^{-1};
UV (H_2O): 238.0, 351.5, 520 nm;
H^1 NMR(D_2O), chemical shifts on the δ scale: 7.10 ($C\underline{H}CO_2H$) 8.00 (ArH) ppm;
C^{13} NMR (D_2O), chemical shifts on the δ scale: 116.04, 118.90, 120.94, 121.27, 122.30, 124.30, 124.68, 126.60, 128.37, 136.48, 136.71, 140.50, 143.93, 144.26, 145.75, 152.01, 154.33, 156.01, 156.67;

Analysis: $(C_{48}H_4O_{40}S_8Na_8)_4-4H_2O$
Found: C 32.74, H 2.50;
Calculated: C 32.58, H 2.71;

D. KY-123 (R_1=OH, R_2=SO_2Na, R_3=H, R_4= $>CH_2$)

KY-1 (2mM) was treated with 5 ml chlorosulfonic acid and the mixture was stirred at 50°C for one-half hour. The resultant mixture was added to 50 g of crushed ice to precipitate the product which was collected by filtration and then washed with ether. The crude sulfonyl chloride product was treated with sodium sulfite (20 mM) in 4 ml water. The reaction mixture was kept slightly alkaline by addition at intervals of small portions of 50% NaOH for 2 days. After solvent removal, ethanol was added to precipitate the product, which was acidified by addition of 50% H_2SO_4, followed by addition of ethanol to precipitate sodium sulfate. The ethanol phase was mixed with ether (1:2, v/v) to precipitate the desired product. Product yield was 39%.

E. KY-147 (R_1=OH, R_2=SO_2NHCH_3, R_3=H, R_4= $>CH_2$)

N-methyl chromotropic acid chloride was formed by reacting chromotropic acid (disodium salt) with thionylchloride in the presence of DMF. The reaction was carried out with stirring at 80°C for 4 hours. After removal of solvent and excess of thionylchloride in vacuo, ether was added to precipitate the chromotropic acid chloride which was subsequently collected by filtration and washed with ether. The crude product was added to 20 ml of methylamine and stirred for 2 hours. After removal of all solvent from the resultant substance, the residue was dissolved in a 200 ml of cold methanol and filtered. The filtrate was added with acetonitrile to precipitate the product chromotropic acid methyl sulfonamide. Yield 56%.

The chromotropic acid methyl sulfonamide (2mM) in 3 ml water was reacted with 37% formaldehyde (1ml) at room temperature for one week. Acetonitrile was added to precipitate the product which was collected by filtration and washed by acetonitrile. Yield was 85%.

F. KY-151 (R_1=OCH_3, R_2=SO_3Na, R_3=H, R_4= $>CH_2$)

KY-1 (50mM) was dissolved in 80 ml of NaOH water solution (0.2M NaOH) and heated to 50°C, dimethylsulfate (0.2M) was added slowly for 1 hour. The mixture was continuously stirred for another 2 hours and left at room temperature for 2 days. Saturated NaCl solution (100 ml) was added to the resultant substance and filtered. The precipitate was washed with ethanol, acetonitrile and ether sequentially. The dry substance was dissolved in 100 ml of methanol and filtered. The filtrate was concentrated and ether was added to precipitate the methyl ether of KT-1.

G. KY-123 (R_1=OH, R_2=SO_2CH_3, R_3=H, R_4= $>CH_2$)

KY-1 from Example 1A was first treated with thionyl chloride to produce chromotropic acid sulfonyl chloride. This compound was reduced by excess sodium sulfite in the presence of sodium bicarbonate to produce the corresponding sodium sulfonate salt of cyclized chromotropic acid (R_2 = SO_2Na). The sulfonate salt was treated with dimethyl sulfate in the presence of $NaHCO_3$, and worked up as as described in Example 1A. Product yield was about 21%.

H. KY-175 (R_1=OH, R_2=SO_3CH_3, R_3=H, R_4= $>CH_2$)

Chromotropic acid was first treated with thionyl chloride to produce chromotropic acid sulfonyl chloride. This compound was then treated with sodium methoxide in methanol in the presence of sodium salt. The product was

worked up as described in Example 1A to form the macrocyclic compound. Product yield was about 29%.

I. KY-270 (R_1=$OCOCH_3$, R_2=SO_3Na, R_3=H, R_4= $>CH_2$)

KY-1 from Example 1A (0.66 mmole) was dissolved in 3 ml water containing 0.1 g NaOH. To this was added 1 g acetyl chloride (13 mmole) and the reaction was allowed to proceed at room temperature overnight with stirring. After solvent removal, 25 ml ethanol was added to precipitate the product. The crude product was dissolved in methanol and filtered. The filtrate was allowed to precipitate, giving a 87% yield.

J. KY-346 (R_1=OH, R_2=SO_3Na, R_3=H, R_4= $-CH_2-N(CH_3)CH_2$)

Chromotropic acid disodium salt, was dissolved in 80 ml of water at a concentration of 50 mM with stirring at 50°C until the solution turned to clear, hexamethylenetetramine (50 mM) was then added to above solution with continuous stirring at the same temperature for additional two hours. At this time, the color of this mixture converted to dark blue. The mixture was allowed to stir at room temperature for 2 days. The resultant dark blue solution was filtered and the filtrate was concentrated, evaporated by flask, which was subsequently treated with 200 ml methanol to precipitate the product KY-346. The yield of KY-346 was 85%. The compound was characterized as follows:

M.P.>300°C;

HPLC in $CH_3CN/MeOH/H_2O/TFA$: 13'07" single peak;

(IR/KBr) = 3425 (OH), 1626 (Ar), 1197, 1052 (SO_3) cm^{-1};

UV (H_2O): 232.0, 377.5 nm

Analysis: $(C_{13}H_{11}O_8NS_2Na_2)_4 \times 12\ H_2O$

Found: C 33.17, H 3.13, N 2.75

Calculated: C 33.98, H 3.59, N 2.96.

Molecular weight: 1668 by gel filtration.

Example 2

Preparation of Phenyl Macrocyclic Compounds

A. Y-49 (R_1=OH, R_2=SO_3H, R_4=-CH_2-, n=4)

4-tert-butylcalix(4)arene (10 g) was treated with 200 ml of concentrated H_2SO_4 at room temperature for 0.5 hour and then at 75-85°C oil bath for another 4 hours. The reaction was completed when no water-insoluble material was detected. The resultant oil was dropped into 500 g of crushed ice and the solution was filtered by reduced pressure. After the water removed away from the filtrate, acetonitrile (500 ml) was added to the residual and allowed to stand for 4 hours to precipitate the crude product which was then collected by filtration and washed with acetonitrile, ethyl acetate and ether. Yield 8 g (73%). The pure product was furnished by recrystallization of the crude compound with methanol-ether or methanol-acetonitrile system. The single crystal compound was also found in the recrystallization process.

Similar methods were used in the synthesis of Y-77 (R_1=OH, R_3=SO_3H, R_4= -CH_2-, n=6) and Y-1 (R_1=OH, R_3=SO_3H, R_4=-CH_2-, n=8).

B. KY-225 (R_1= -OH, =O), R_2=SO_3H, R_4= >CH_2, ≥ CH, n=4)

4-tert-Butylcalix(4)arene (1 g) was treated with 10 ml of 95-98% sulfuric acid at room temperature for 0.5 hours then at 160°C for 5 minutes. After the resultant mixture was cool, the mixture was poured slowly into 100 ml of crushed ice and filtrated. The solution was evaporated and the residual was added with 300 ml acetonitrile to produce great amount of precipitate which was collected by filtration and washed with acetonitrile. The crude product was dissolved in 20 ml methanol and the product was precipitated by addition of diethyl ether. Yield was 84%.

Similar methods were used in the synthesis of Y-48 (R_1= -OH or =O, R_3=SO_2H, R_4= $-CH_2-$, n=6) and Y-226 (R_1= -OH or =O, R_2=SO_3H, R_4= $-CH_2-$, n=8).

C. O-Acetylate of Y-1 (R_1= $-OCOCH_3$, R_2=SO_3Na, R_4= $>CH_2$, n=8)

Under nitrogen, Y-1 (0.4 g) was refluxed in a stirring mixture of NaOAc (305 mg) and acetic anhydride (20 ml) for 3 days. After cooling to room temperature, the suspension was filtered. The solid was washed three times with ether (25 ml) and dried in vacuo. The resulting solid was sonicated in a mixture of MeOH (50 ml) and activated charcoal (150 mg), filtered, and the black precipitate was washed twice with MeOH (10 ml). The filtrate was concentrated in vacuo. The resulting residue was recrystallized from MeOH/acetonitrile mixture. The product (240 mg) was obtained after filtration and lyophilization.

^{13}CNMR (D_2O, δ): 173.9, 151.6, 144.1, 135.6, 130.1, 34.2, and 22.4.

D. Y-78 (R_1= -OH, R_2=SO_2NH_2, R_4= $>CH_2$, n=8)

Under nitrogen, Y-1 (1 g) is heated at 60-70°C with chlorosulfonic acid (20 ml) for 1 hour. After cooling to room temperature, the oily material is poured into ice water, and the precipitate is filtered. After washing the precipitate with cold water, the crude product is dissolved in 100 ml of 25% ammonium water solution and allowed to react for 2 hours at room temperature. The mixture is concentrated in vacuo and the remaining oil is dissolved in a small amount of water and filtered. The product is precipitated by adding acetonitrile to the filtrate and collected by filtration and washing with acetonitrile.

E. Glycyl sulfonamide of Y-1 (R_1= -OH, R_2=$SO_2NHCH_2CO_2H$, R_4= $>CH_2$, n=8)

Under nitrogen, Y-1 (1 g) is heated at 60-70°C with chlorosulfonic acid (20 ml) for 1 hour. After cooling to room temperature, the oily material is poured into ice water, and the precipitate is filtered. After washing the precipitate with cold water, the material is added to 50 ml of solution containing 5.7 g glycine and 2.1 g NaOH, and stirred for 2 hours at room temperature. After removal of all solvent from the resultant substance, the residue is dissolved in a 200 ml of cold methanol and filtered. The filtrate is added with acetonitrile to precipitate the product.

F. Acetyl-Bridged Y-49 (R_1= -OH, R_2=SO_3H, R_4= $-CHCO_2H$, n=4)

4.3 g of p-hydroxybenzenesulfonic acid was treated with g gram of glyoxylic acid in 30 ml 18% conc. HCl for 2 hours at 100°C. After the reaction product was dried under reduced pressure, 50 ml of methanol was added and insoluble impurities were removed by filtration. The product was precipitated from the filtrate by addition of ether then collected by filtration and dried in vacuo.

G. Toluene Sulfonyl Ester of Y-49 (R_1= $-SO_3C_6H_4CH_3$, R_2=SO_3H, R_4= $>CHCO_2H$, n=4)

Under nitrogen is added toluenesulfonyl chloride (1.9 g) to a suspension of dry sodium carbonate (1.06 g), dry dimethylformamide (10 ml) and Y-49 (0.75 g). After an overnight reflux, the resulting mixture is cooled to room temperature and filtered. The filtrate is diluted with ether to precipitate out the crude product. Recrystallization from acetonitrile/ether solvent provided the product.

H. Carboxylic Acid Derivative of Y-49 (R_1= $-CO_2H$, R_2=SO_3H, R_4= $>CHCO_2H$, n=4).

Under nitrogen, trifluoromethanesulfonic anhydride (1.0 ml) is added to ice cold dry dichloromethane solution (10 ml) of 2,6, di-tert-butyl-4-methylpyridine (1.25 g) and 4-tert-butylcalix[4]arene (0.65 g). After overnight stirring at room temperature, the mixture is diluted with pentane (10 ml) and filtered. The filtrate is extracted with ice cold 1N aqueous NaOH solution, ice cold 1N aqueous HCl solution, then saturated aqueous NaCl solution, dried over anhydrous sodium sulfate, filtered through a pad of silica gel and concentrated in vacuo. The residue is dissolved in a mixture of dry diisapropylethylamine (10 ml), trimethylsilyl cyanide (0.5 ml) and palladium tetrakis-triphenylphosphine (20 mg). After an overnight reflux under nitrogen and then cooling to room temperature, ether (50 ml) was added and the resulting suspension was filtered. After concentration of the filtrate in vacuo and silica gel chromatography (hexane/ethyl acetate eluent), the cyano intermediate is heated at 80°C with concentrated sulfuric acid (10 ml) for 3 hours, diluted with water (10 ml) and refluxed overnight. After cooling to room temperature, the resulting mix is added to charcoal (0.5 g) and ice (50 g). After filtration, the resulting filtrate is concentrated in vacuo to ca 15 ml in volume and the resulting solid was filtered. The solid is dissolved in a minimal amount of methanol and precipitated out by adding ether. Final purification by reverse phase C18 chromatography (methanol/water eluent) provide the product.

I. Methyl Ether of Y-1 (R_1 =OMe, R_2=SO_3Na, R_4= $>CH_2$, n=8).

Iodomethane (0.58 ml) was added to a heated (50°C)

mixture of Y-1 (447 mg), NaOH (6 N in water, 1.53 ml), and dimethylsulfoxide (9 ml) for 20 hours. The resulting mix was added dropwise into stirring absolute ethanol (100 ml). The resulting suspension was centrifuged (9,000 rpm, 20 minutes), and then the supernatant was removed. Twice, the resulting solid was dissolved in water (6 ml), and the resulting solution was treated as above with ethanol, centrifuged, and the supernatant removed. The remaining solid was lyophilized to yield the product (420 mg).

^{13}CNMR (D_2O, δ): 161.2, 140.9, 137.6, 129.5, 63.6, and 33.5.

Example 3

Preparation of Aryl-Bridged Macrocyclic Compound

Chromotropic acid, disodium (10 g) in 55 ml of water was treated with 22 ml of 30 ml 37% HCl. To this solution, 1,2-benzenedimethanol (5 g) in 55 ml of acetic acid was added and this reaction was carried at reflex for 6 hours. After filtration of the resultant mixture, acetonitrile (500 ml) was added to precipitate the crude product and collected it by filtration. The crude compound was further purified by column chromatographic purification on LH-20 resin and elution with ethanol.

Example 4

Anticoagulant Activity Assays

A. Preparation of Blood samples

Venous blood samples were taken using clean venipuncture procedures. Samples were collected in collection tubes (VACUTAINER™) containing sodium citrate such that 9 parts of blood were added to 1 part of 3.8% sodium citrate, and placed in an icebath. Prior to centrifugation, blood samples were checked for the

presence of clots, and any tubes containing clots were discarded.

Samples were centrifuged at 1500 x g for 15 minutes in a refrigerated clinical centrifuge. Plasma was removed from the sample using a non-wettable plastic pipet and stored in a non-wettable plastic stoppered tube, at 4°C. Plasma samples showing evidence of hemolysis were discarded. Plasma samples were then placed in an icebath for testing within 8 hours of collection, or were alternatively frozen at -20° for testing within 1 week of collection. All in vivo specimens after drug administration were collected into VACUTAINER® coagulation tubes for assays of PT, APTT and fibrinogen within 8 hours without freezing.

B. Prothrombin Time Assay (PT)

Plasma samples collected as described in Example 4A, were pre-warmed to 37° in a waterbath, then 0.1 ml aliquots above were forcibly added to tubes containing 0.2 ml thromboplastin-calcium reagent (Dade® Thromboplastin•C, Becton Dickinson), reconstituted and stored according to manufacturers directions, pre-warmed and resting in a 37° waterbath. Each tube was timed individually while being mixed gently, using the manual tilt tube method. Time until visible clot formation was obtained for each sample. All samples were assayed in triplicate, and the clotting times (PT times) were averaged.

C. Activated Partial Thromboplastin Time (APTT)

Plasma samples were collected as described in Example 4A and stored in an icebath until testing. 0.1 ml of plasma sample was mixed with 0.1 ml of partial thromboplastin (Actin® Activated Cephaloplastin Reagent; Becton Dickinson), reconstituted and handled according to

manufacturer's instructions, in a 13 x 100mm polyethylene tube. The tube containing the mixture was placed in a 37° water bath for 3 minutes, prior to addition of 0.2 ml of pre-warmed (37°) 0.025 M calcium chloride). The tube was then tilted gently at 5 second intervals, for a total of 20 seconds at 37°, then removed from the water bath and the periodic tilting continued as the sample was observed for fibrin web formation. All samples were assayed in duplicate.

D. Plasma Recalcification Time (Plasma Clotting Time)

Blood was collected in 8% sodium citrate and centrifuged for at least 20 minutes at 1500 x g to obtain platelet-poor plasma. A 50 microliter aliquot of plasma was mixed with 50 microliters of physiological saline solution, and the mixture was placed in a test tube at room-temperature. Twenty-five microliters of pre-warmed (37°) 1% (1g/100ml) calcium chloride was added to the test tube with gentle mixing. The mixture was then tilted at 1 minute intervals and observed for clot formation.

E. Fibrinogen Assay

Blood was collected as described in Example 1A. Clotting time was determined after addition of thrombin reagent (Data-Fi® Thrombin Reagent, Baxter Healthcare Corp., Miami, FL; reconstituted according to manufacturer's instructions) a dilute plasma sample. This time was compared to a standard curve of clotting times for samples containing known amounts of fibrinogen, to determine fibrinogen concentration of the dilute sample.

F. Reptilase Assay (Atroxin Time)

Blood samples were centrifuged to produce platelet poor plasma (1500 x g, 15 min.), as described in Example 4A. Plasma samples (0.2 ml) were incubated at 37° for 5 minutes. ATROXIN® (Sigma Chemical Co., St. Louis, MO), prewarmed to 37°, was added as a 0.1 ml aliquot to each sample tube with mixing to initiate the reaction. Time to clotting was recorded as Atroxin time.

G. Thrombin Time Assay (TT)

Thrombin solution was prepared by diluting concentrated stock human a-thrombin (4270 u/ml) into barbital buffered saline, pH 7.35 to achieve a 10X concentrated working stock. The final concentration of thrombin to be used in standard assays was determined by testing serial dilutions of concentrated stock a-thrombin for its ability to produce a clotting time of 20 sec ± 0.5 sec. in control platelet poor plasma samples. Platelet poor plasma was prepared as described in Example 4A, and prewarmed at 37° in 0.18 ml aliquots. Test compounds or saline were added to the plasma samples to produce a final volume of 0.2 ml. The reaction was initiated by addition of 10 μl of 10-fold concentrated stock purified human a-thrombin to each sample. Incubation was continued at 37° with gentle sweeping of each sample with a wire loop 2 times per second, until a clot formed in the loop. Time to clot formation was recorded for all samples.

H. Platelet Aggregation

Blood samples were drawn with a plastic syringe and transferred to plastic test tubes containing a sufficient volume of sodium citrate to produce a final concentration of 0.011 M sodium citrate in the sample. Samples of platelet rich plasma (PRP) and platelet poor plasma (PPP)

were prepared from each sample by first centrifuging the sample at 150 x g for 5 minutes at room temperature and collecting the red blood cell free supernatant (PRP), then centrifuging the remaining blood at 1500 x g for 15 minutes to obtain the PPP supernatant. PRP and PPP were held in tightly capped plastic tubes until testing. PRP was verified by performing a platelet count and determining that platelet levels were between 200,000 and 300,000 per μl PRP. If necessary, dilution of PRP samples with PPP was made to dilute platelets to this level.

A control 0.5 ml sample of PPP was transferred to an aggregometer cuvette. Several 0.45 ml samples of room temperature PRP were transferred to separate cuvettes. A baseline reading of the PPP sample was obtained by placing the PPP in the aggregometer and incubating at 37° according to aggregometer manufacturer's instructions. A PRP sample was then placed in the aggregometer and allowed to equilibrate for 2 minutes, prior to addition of test reagent contained in 0.05 ml saline. Percent aggregation values were obtained for each sample.

I. Plasmin Assay

Plasmin chromogenic assays were carried out using a standard clinical protocol, at the Stanford University Blood Bank.

Example 5

Effect of macrocyclic compounds on plasma recalcification (clotting time)

Venous blood samples were collected from rat tail vein into tubes containing sodium citrate (8%), and plasma was prepared, using procedures described in Example 1A. Recalcification of plasma was carried out, as described in Example 1D in a total volume of 0.125 ml. To 50 μl

plasma was added 50 μl saline or compound dissolved in saline. Plasma recalcification was initiated by addition of 25 μl 1% (wt/vol) calcium chloride. Effects of these studies are shown in Table 3, in which each compound was tested at a final concentration of 12.5, 25, and 50 μg/ml. Clotting time values are expressed as heparin equivalent weights, in micrograms. 1 USP unit = 6.4 μg heparin.

Example 6

Effect of increasing concentrations of KY-1, Y-1 and Y-49 on Prothrombin Time (PT) in vitro

KY-1, Y-1 and Y-49 were tested in a PT assay using platelet poor plasma prepared from human blood, similar to that described in Example 4B. In this assay, plasma samples (0.1 ml) were pre-mixed with 10 μl saline containing varying amounts of test compound (0-250 μg/ml, final concentrations). The resulting mixed aliquots were forcibly added to tubes containing 0.2 ml pre-warmed (37°) Thromboplastin-calcium reagent (Dade® Thromboplastin•C, Baxter Healthcare Corp., Hayward, CA). Each tube was timed individually for clot formation while subjected to gentle mixing, using the manual tilt tube method. Time to clot formation was recorded as PT for each sample.

Example 7

Effect of KY-1, Y-49 and Y-1 on APTT

KY-1, Y-1 and Y-49 were tested in an APTT assay using platelet poor plasma prepared from human blood, similar to that described in Example 4C. Plasma samples were collected as described in Example 4A. Plasma samples (0.1 ml) were pre-mixed with 0-364 μg of test compound in 10 μl saline. The mixed samples were added to 0.1 ml of APTT reagent (Automated APTT®, Organon Teknika Corp.,

Durham, NC), prior to addition of 0.2 ml 0.025 M calcium chloride, pre-warmed to 37° on a fibrometer plate. The sample was timed for formation of fibrin web.

Example 8

Effect of KY-1, Y-49 and Y-1 on Thrombin Time (TT)

Compounds were tested for effects on thrombin time (TT). Platelet poor human plasma was prewarmed at 37° in 0.18 ml aliquots in the presence of 20 μl of test compound (0-190 μg/ml final concentration). The reaction was initiated by addition of 10 μl (42u/ml) purified human a-thrombin (amount calibrated to give a TT of 20 sec. in untreated human platelet poor plasma). Time to clot formation was recorded for all samples.

Example 9

Reptilase Assay (Atroxin Time)

Samples of human platelet poor plasma (0.2 ml) were incubated at 37° with 10 μl aliquots of saline containing test compound (final concentration, 0-900 μg/ml) for 5 minutes. ATROXIN® (Sigma Chemical Co., St. Louis, MO), prewarmed to 37°, was added as a 0.1 ml aliquot to each sample tube to initiate the reaction. Time to clotting was recorded as Atroxin time.

Example 10

Effect of KY-1 and Y-1 on Plasmin Activity

KY-1 and Y-1 were tested for effects on plasmin chromogenic activity. KY-1 was tested at concentrations of 20.3, 40.6, and 81.1 μg/ml final concentration, and Y-1 was tested at final concentrations of 9.4 and 18.8 μg/ml. By way of comparison, heparin was tested at a final concentration of 0.41 μg/ml. Results are shown in Figures 22 A-C.

Example 12

Effect of intravenous administration of macrocyclic compounds on PT, APTT and fibrinogen content of rat plasma

Macrocyclic compounds at various concentrations were dissolved in phosphate buffered saline (pH 7.4) and administered to rats at 2.5, .5, or 25 mg/kg, intravenously. At various times after administration, a blood sample was taken from each rat, plasma prepared, as described in Example 4A, and determinations of PT, APTT, and fibrinogen content made, as described in Examples 4B, 4C and 4E, respectively.

Example 13

Effect of oral Y-1 on Plasma Clotting Time

Female Swiss-Webster mice (27-28 g each) were each given 2 doses (500 or 625 mg/kg) of compound or saline (PBS) at 30 minute intervals by gastric gavage. Blood samples were collected into 8% citrate via retro-orbital venipuncture 2.5 hours following the initial dosing. Blood plasma was obtained, processed as described in Example 4A, and assayed for plasma clotting time as described in Example 4C. For comparison, Y-1 (12 or 20 μg/ml) was added to plasma samples from saline treated control animals, and samples were tested for clotting time as described in Example 5. Results of this assay are shown in Table 4.

Example 14

Time course of effect of orally administered Y-1 on PT and APTT

Rats were given Y-1 at a dose of 450 mg/kg by gastric gavage. Arterial blood samples were withdrawn at 0.5, 4, 8, 16, and 24 hours through a cannula inserted in the

left carotid artery, with tip extending to the descending aorta. Plasma samples were prepared as described in Example 4A, and tested for PT and APTT, as described in Examples 4B and 4C. An additional dose of 225 mg/kg was administered to a subgroup of animals 23 hours after the initial dosing, and plasma from these animals was also tested at 24 hours. Results are shown in Table 6.

Example 15

Effect of intravenous Y-1 on PT and APTT

Male Sprague-Dawley rats (2/dose) were given 2.5 mg/kg or 5 mg/kg Y-1 intravenously in the lateral tail vein. At various time intervals following injection, rats (4/time period) were bled through a cannula inserted in the cartoid artery to the descending aorta, and the blood was processed to obtain plasma, as described in Example 4A. An APTT assay was carried out on each plasma sample.

Example 16

Effect of KY-1, Y-1, and Y-49 on Platelet Aggregation

General platelet aggregation assay procedures were used as described in Example 4I. Collagen dose was titrated to give maximal response to platelet aggregation using citrated platelet-rich plasma. KY-1 and Y-49 at 24 μg/ml and 48 μg/ml concentrations had no effect on collagen-induced aggregation (0.47 μg/ml). Y-1 at 24 μg/ml had a slight inhibitory effect while at 48 μg/ml showed significant inhibition.

IT IS CLAIMED:

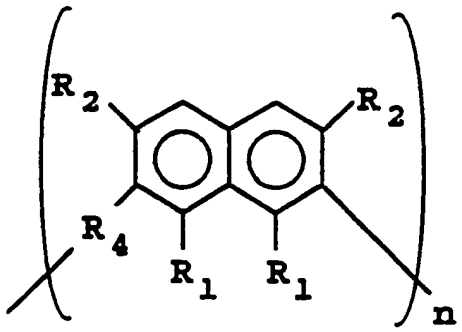
1. A pharmaceutical composition effective in inhibiting coagulation in a human subject, comprising a macrocyclic compound composed of aryl ring subunits which are connected by ring-attached bridge linkages which form a continuous chain of connected atoms making up the backbone of the macrocycle, and which contain sulfonic-acid derived substituents on non-backbone atoms for the aryl subunits, and

a pharmaceutically acceptable carrier in which the peptide is carried.

2. The composition of claim 1, wherein the sulfonic acid-derived substituents are selected from the group consisting of sulfonic acid, sulfonate salt, sulfinic acid, sulfinate salt, a sulfone, and a sulfonamide.

3. The composition of claim 1, wherein the aryl ring subunits are selected from the group consisting of (a) naphthalene subunits with 1- and 8-position polar groups, and 3- and 6-position sulfonic acid-derived substituents, (b) phenyl subunits with 1-position polar groups, and 4-position sulfonic acid derived groups, and (c) a mixture of (a) and (b), where the bridge linkages are between the 2 ring-carbon position of one naphthalene or phenyl group, and the 7 ring-carbon group of an adjacent naphthalene group or 6 ring-carbon position of an adjacent phenyl group.

4. The composition of claim 3, wherein the macrocyclic compound has the form:

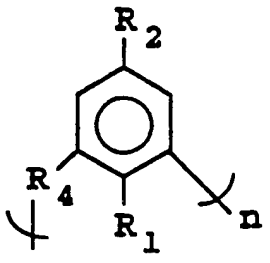


where R_2 is sulfonic acid, sulfonate salt, sulfinic acid, sulfinate salt, a sulfone, or a sulfonamide, R_1 is OH, =O, an alkyl or aryl ether, ester, or acid, or a mixture thereof, R_4 is >CHR", or >CR", where R" is H or carboxylic acid group and n = 4, 6, or 8.

5. The composition of claim 4, wherein R_2 is sulfonic acid or a sulfonate salt.

6. The composition of claim 4, wherein R_2 is an alkyl sulfone or SO_2NHR, where NHR is NH_2, NHOH or an amino acid.

7. The composition of claim 3, wherein the compound has the form,



where R_2 is sulfonic acid, sulfonate salt, sulfinic acid, sulfinate salt, a sulfone, or a sulfonamide, R_1 is OH, =O, an alkyl or aryl ether, ester, or acid, or a mixture thereof, R_4 is >CHR", or ≥CR", where R is H or carboxylic acid group and n = 4, 6, or 8.

8. The composition of claim 7, wherein R_1 is selected from the group consisting of $-OCOCH_3$, $-SO_3C_6H_4CH_3$, -COOH, and OMe.

9. The composition of claim 7, wherein R_2 is an alkyl sulfone or SO_2NHR, where NHR is NH_2, NHOH or an amino acid.

10. The composition of claim 7, wherein some of the R_1 groups are =O.

11. The composition of claim 1, wherein the composition is formulated to produce a maximum blood concentration between about 10-100 μg/ml of compound, following oral administration.

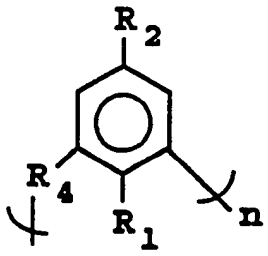
12. A pharmaceutical composition effective in inhibiting blood coagulation in a human subject, comprising a macrocyclic biocompatible polymer composed of at least six regularly spaced sulfonic-acid derived substituents selected from the group consisting of an alkyl sulfone, and a sulfonamide of the form SO_2NHR, where NHR is NH_2, NHOH, or an amino acid, and a pharmaceutically acceptable carrier in which the peptide is carried.

13. The composition of claim 12, wherein the polymer is a macrocyclic compound composed aryl ring subunits which are connected by ring-attached bridge linkages

which form a continuous chain of connected atoms making up the backbone of the macrocycle, and which contain the sulfonic-acid derived substituents on non-backbone atoms of the aryl subunits.

14. The composition of claim 13, wherein the macrocyclic compounds is composed of at least six phenyl-ring subunits, each containing one such sulfonic-acid derived substituent.

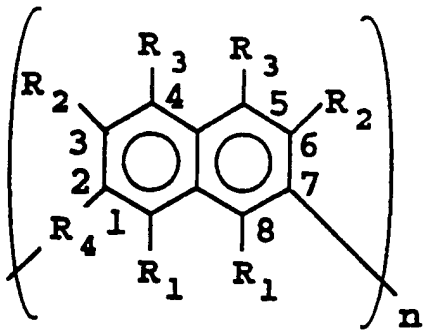
15. A macrocyclic compound which has the form:



where R_2 is sulfonic acid, sulfonate salt, sulfinic acid, sulfinate salt, a sulfone, or a sulfonamide, R_1 is =O, and -OH, an alkyl or aryl ether, ester, or acid, or a mixture thereof, R_4 is >CHR" and >CR", where R" is H or carboxylic acid group and n = 4, 6, or 8.

16. The compound of claim 15, wherein R_1 is selected from the group consisting of $-OCOCH_3$, $-SO_3C_6H_4CH_3$, -COOH, and OMe.

17. The compound of claim 15, wherein R_2 is an alkyl sulfone or SO_2NHR, where NHR is NH_2, NHOH or an amino acid.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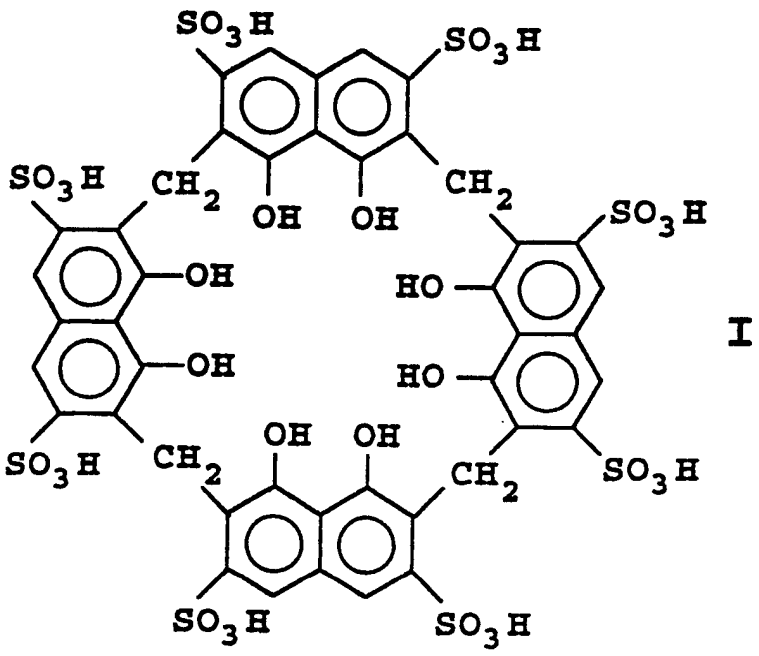
R3
R2
4
5
R2
3
6
2
7
R4
1
8
R1
R1
n

Fig. 1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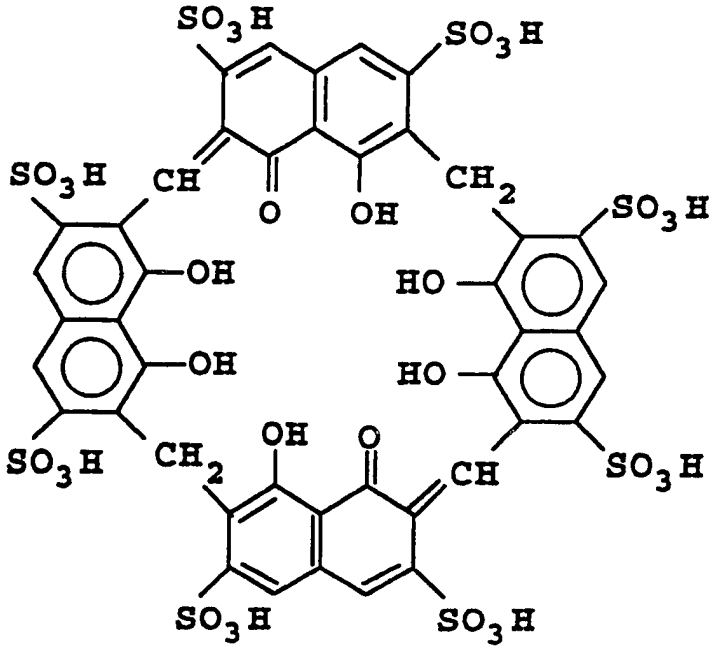
SO3H
SO3H

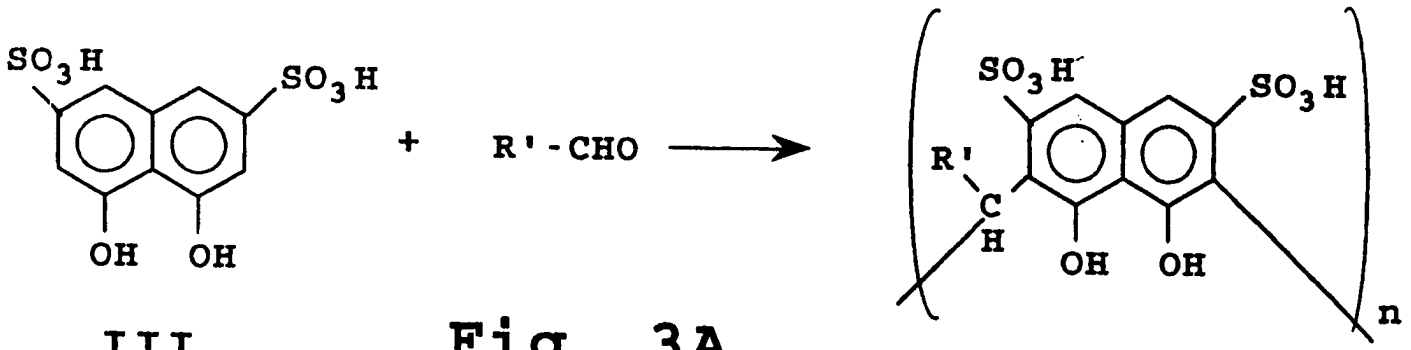
I

Fig. 2A



II

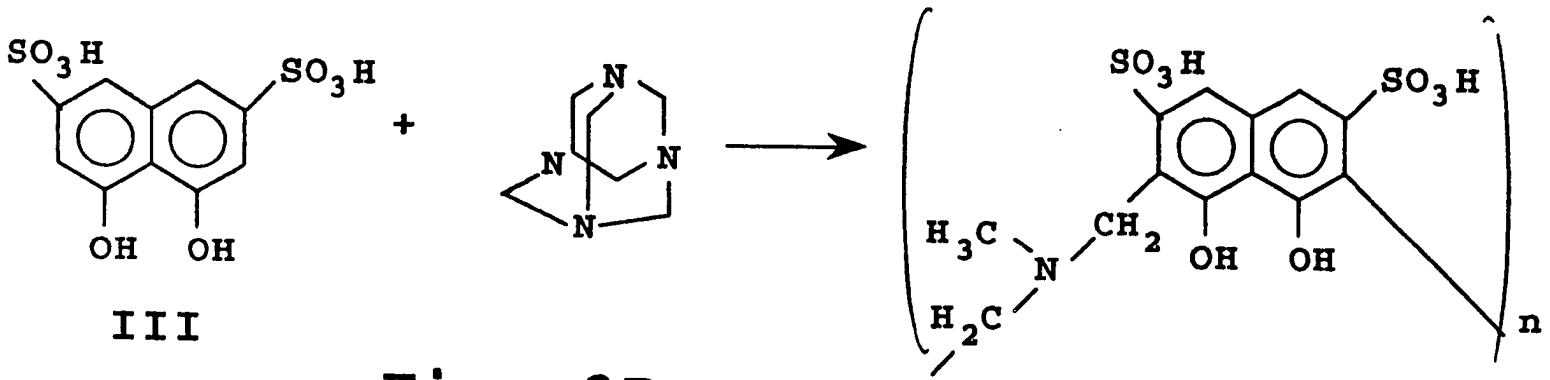
Fig. 2B



III

Fig. 3A

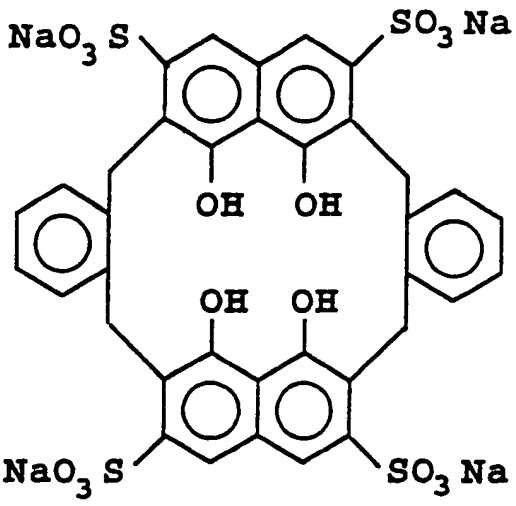
IV



III

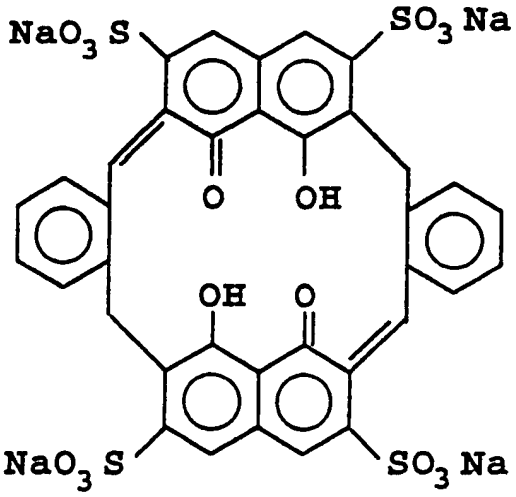
Fig. 3B

V



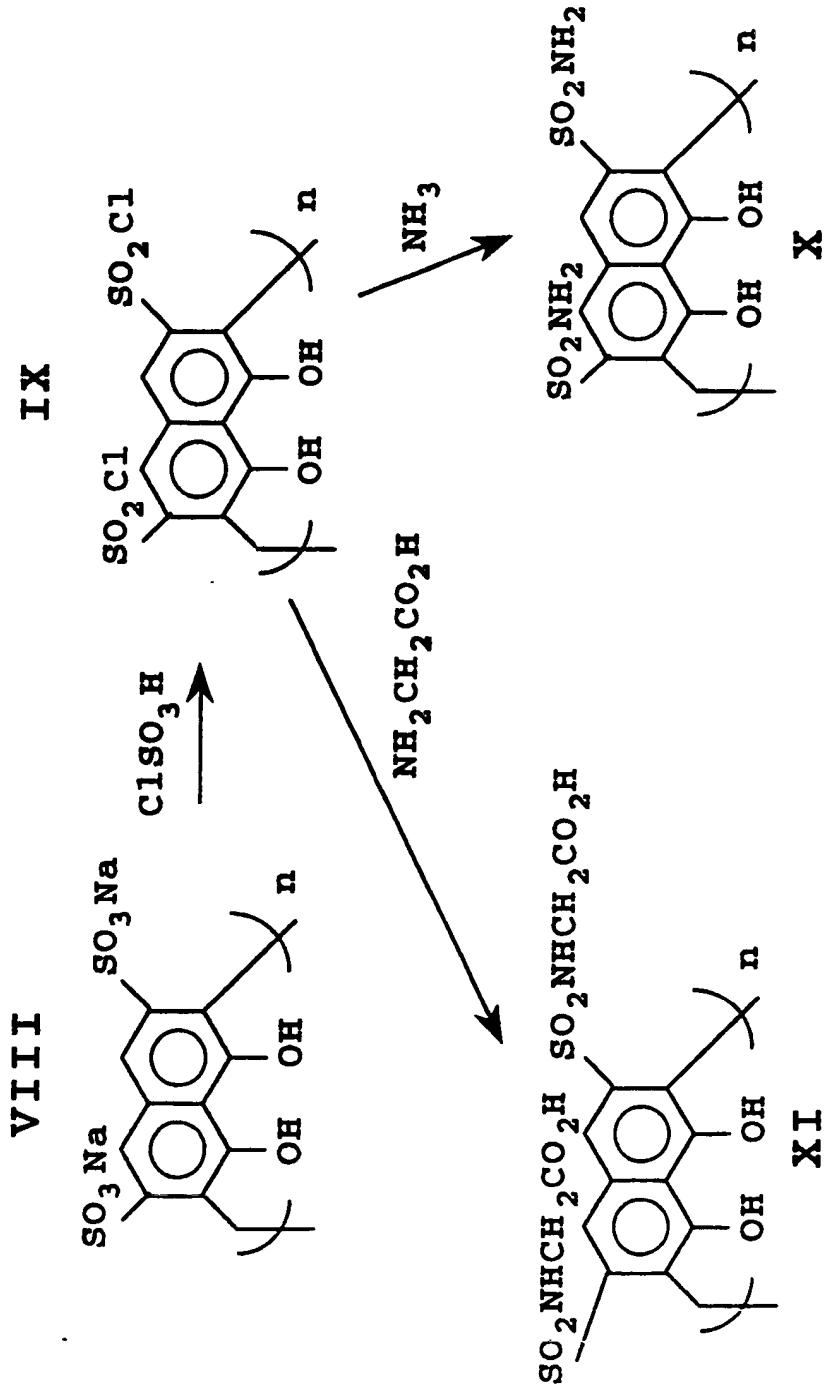
VI

Fig. 4A



VII

Fig. 4B


VIII
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X
XI
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SO_2Cl
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OH
n
$ClSO_3H$
NH_3
$NH_2CH_2CO_2H$

Fig. 5

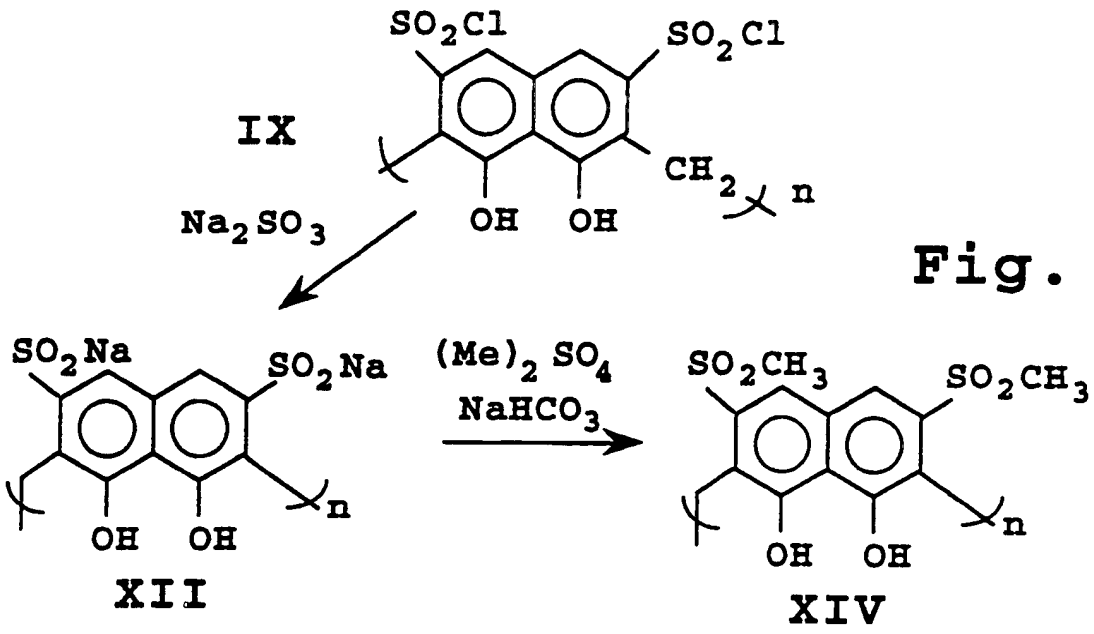


Fig. 6

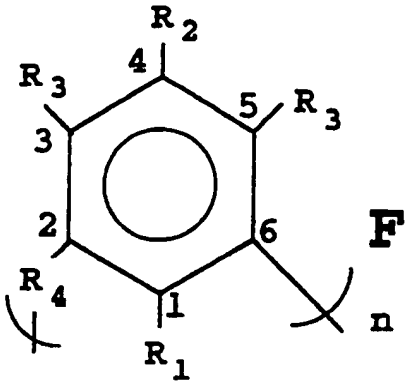


Fig. 7

Fig. 8

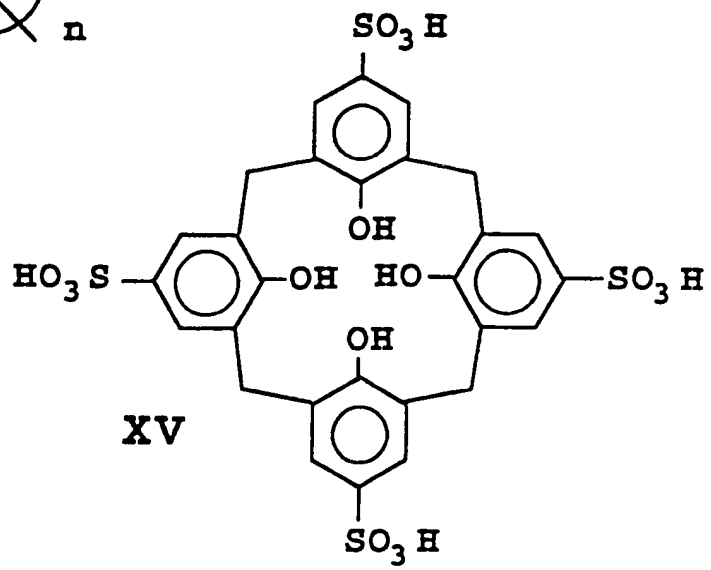
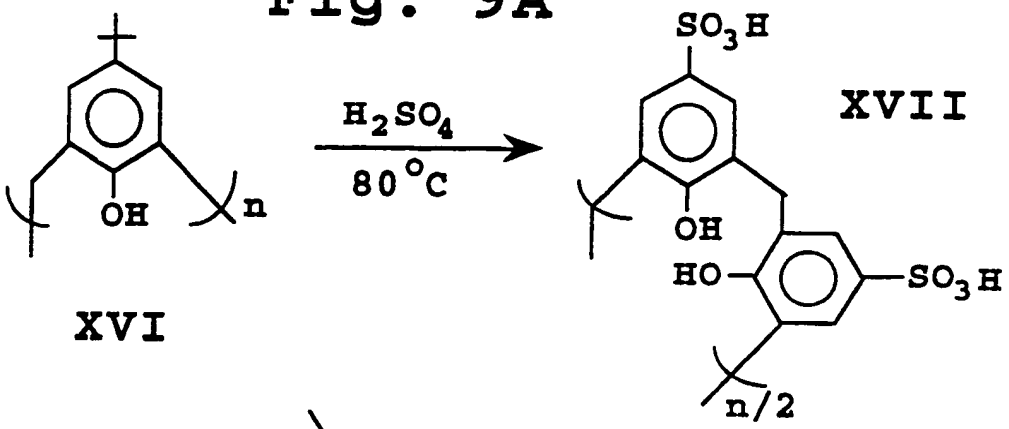


Fig. 9A



XVI

XVII

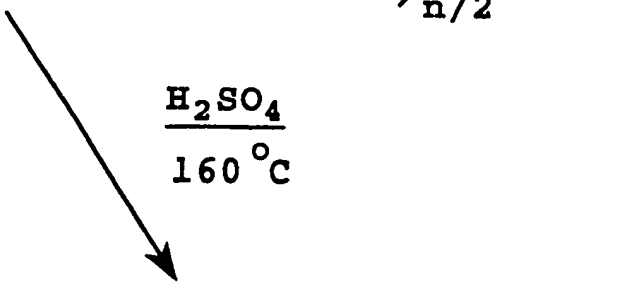
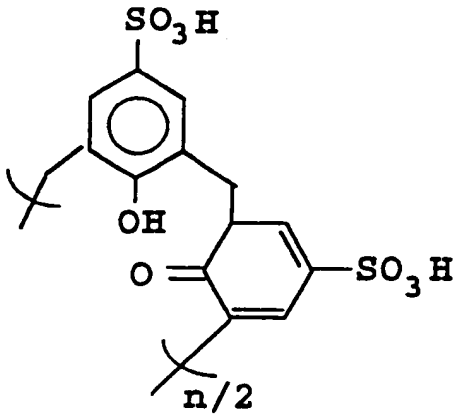
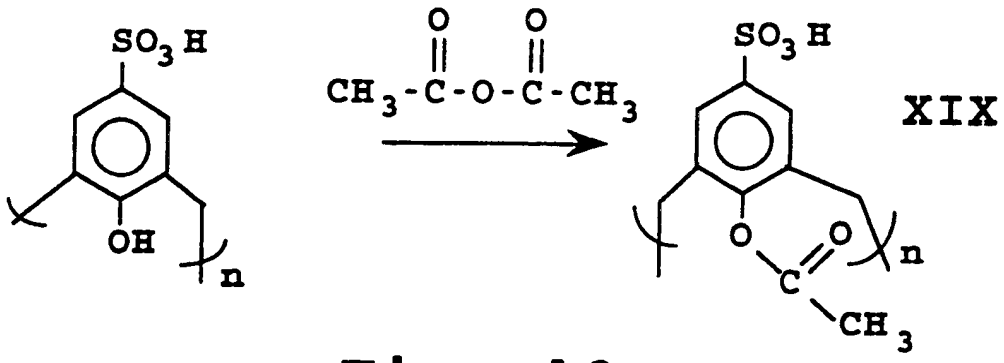


Fig. 9B



XVIII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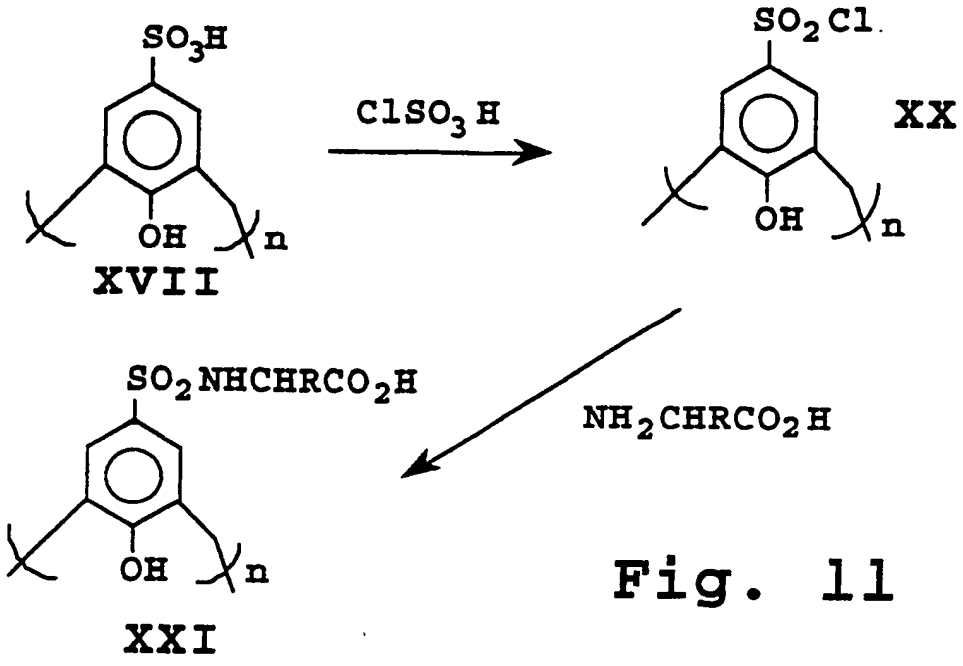
O
O
SO3H
XIX
O
O
n
C
CH3

Fig. 10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SO2Cl
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XXI

Fig. 11

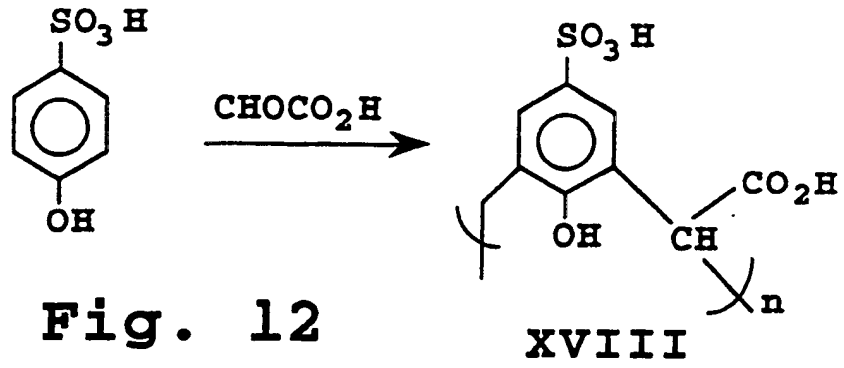
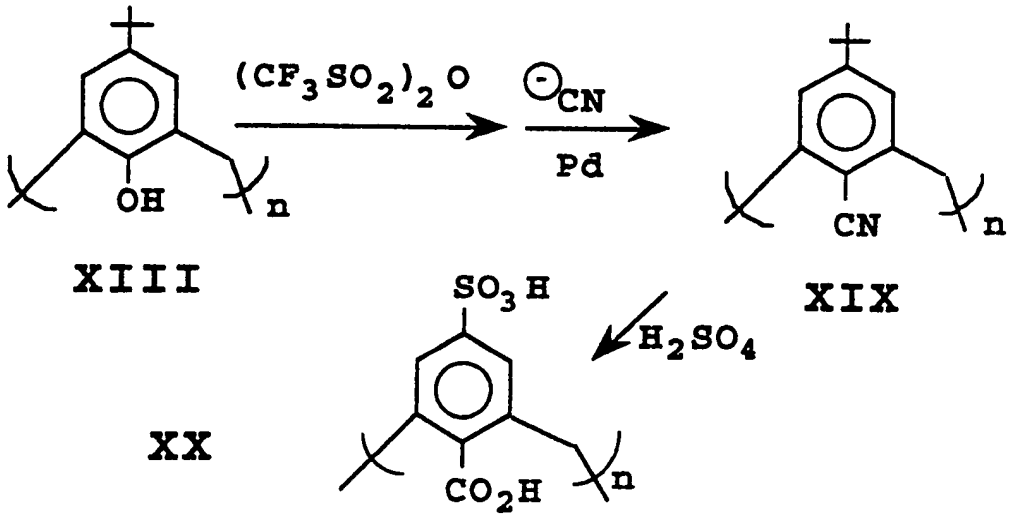


Fig. 12

XVIII



XIII

XIX

XX

Fig. 13

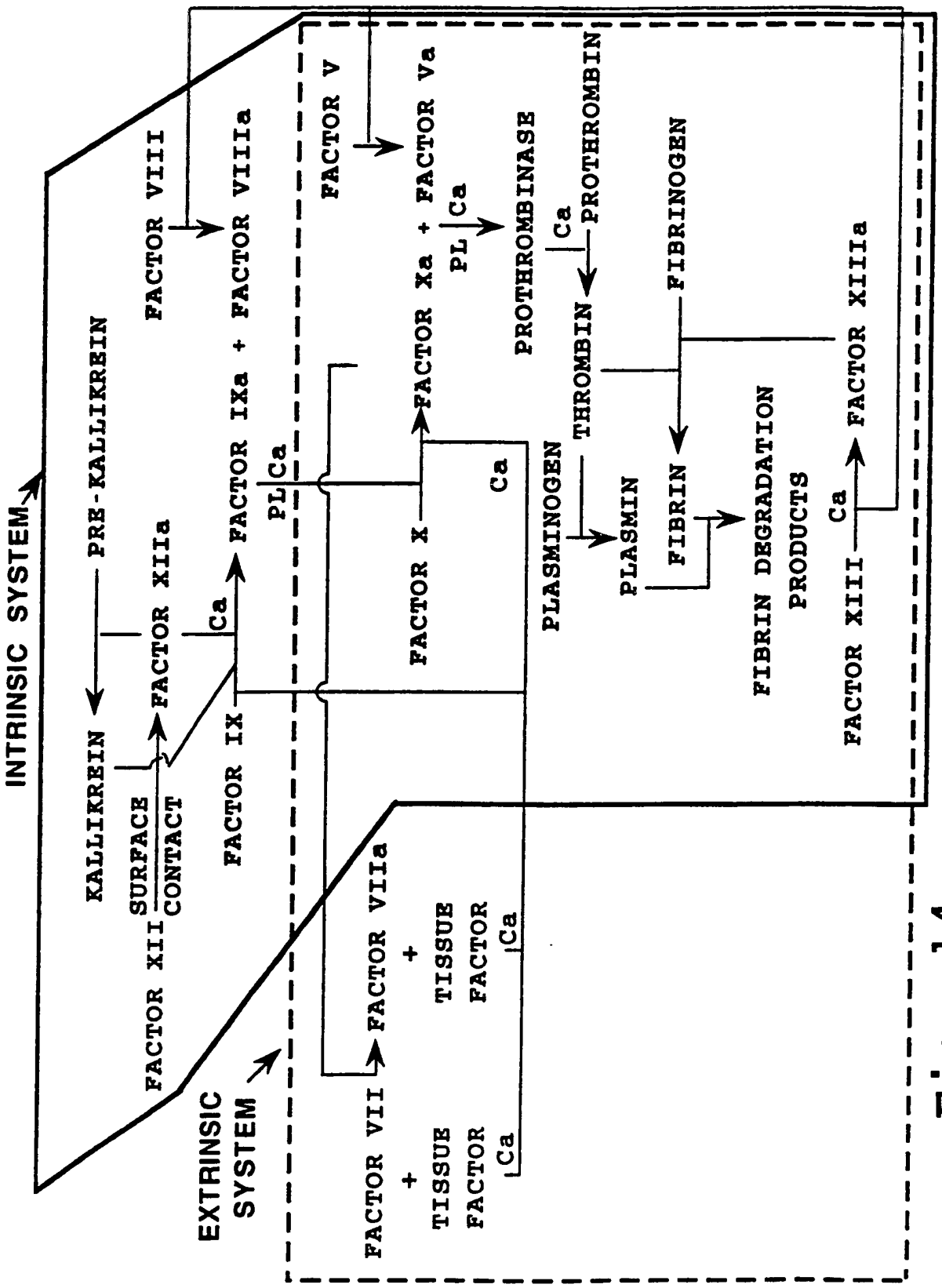

INTRINSIC SYSTEM
EXTRINSIC SYSTEM
PRE-KALLIKREIN
KALLIKREIN
FACTOR XII
SURFACE CONTACT
FACTOR XIIa
FACTOR IX
FACTOR IXa + FACTOR VIIIa
FACTOR VIII
FACTOR V
FACTOR Xa + FACTOR Va
FACTOR X
PROTHROMBINASE
PROTHROMBIN
THROMBIN
PLASMINOGEN
PLASMIN
FIBRINOGEN
FIBRIN
FIBRIN DEGRADATION PRODUCTS
FACTOR XIII
FACTOR XIIIa
FACTOR VII
FACTOR VIIa
TISSUE FACTOR
Ca
PL

Fig. 14

Fig. 15

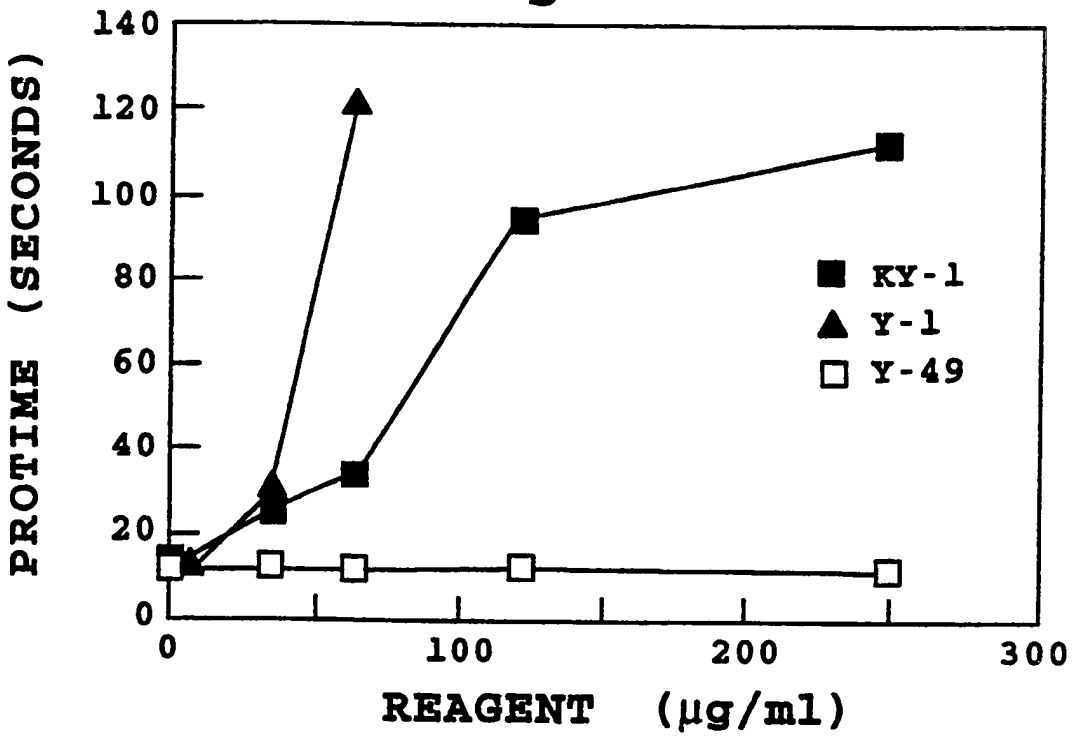
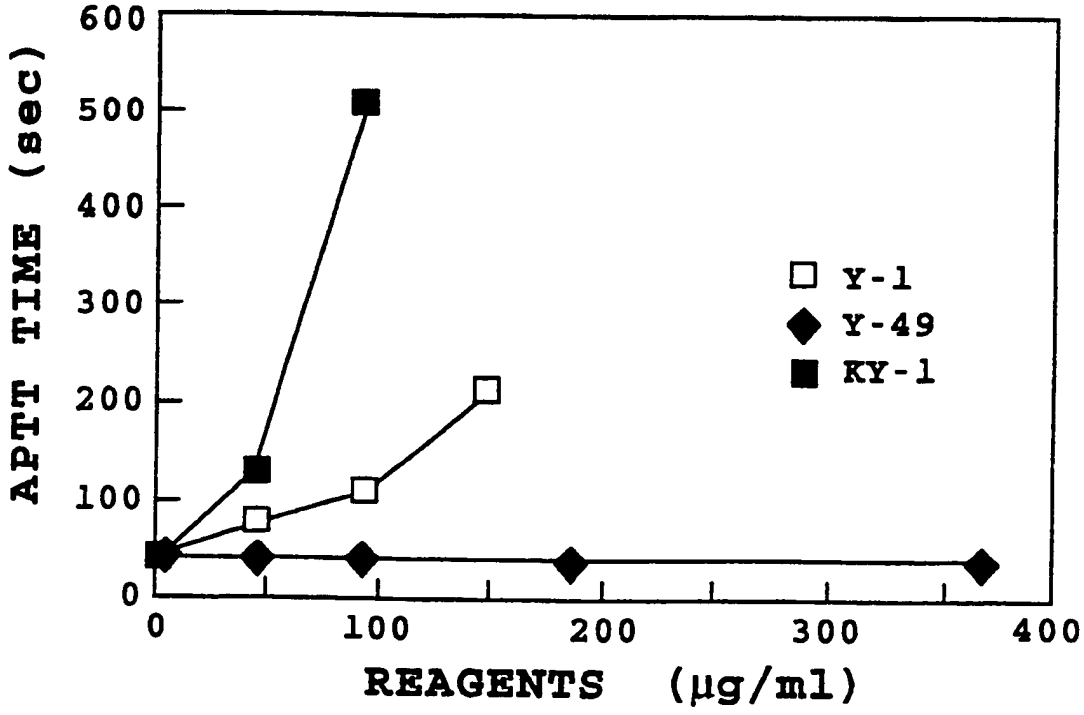

PROTIME (SECONDS)
140
120
100
80
60
40
20
0
0
100
200
300
REAGENT (µg/ml)
KY-1
Y-1
Y-49

Fig. 16


APTT TIME (sec)
600
500
400
300
200
100
0
0
100
200
300
400
REAGENTS (µg/ml)
Y-1
Y-49
KY-1

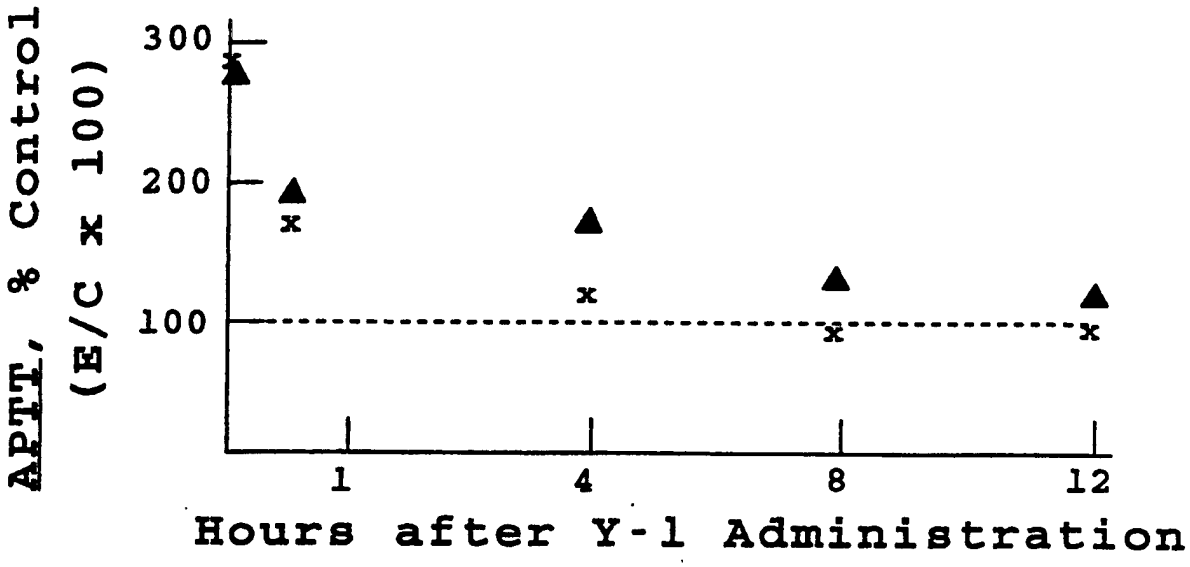

APTT, % Control
(E/C x 100)
300
200
100
1
4
8
12
Hours after Y-1 Administration

Fig. 17

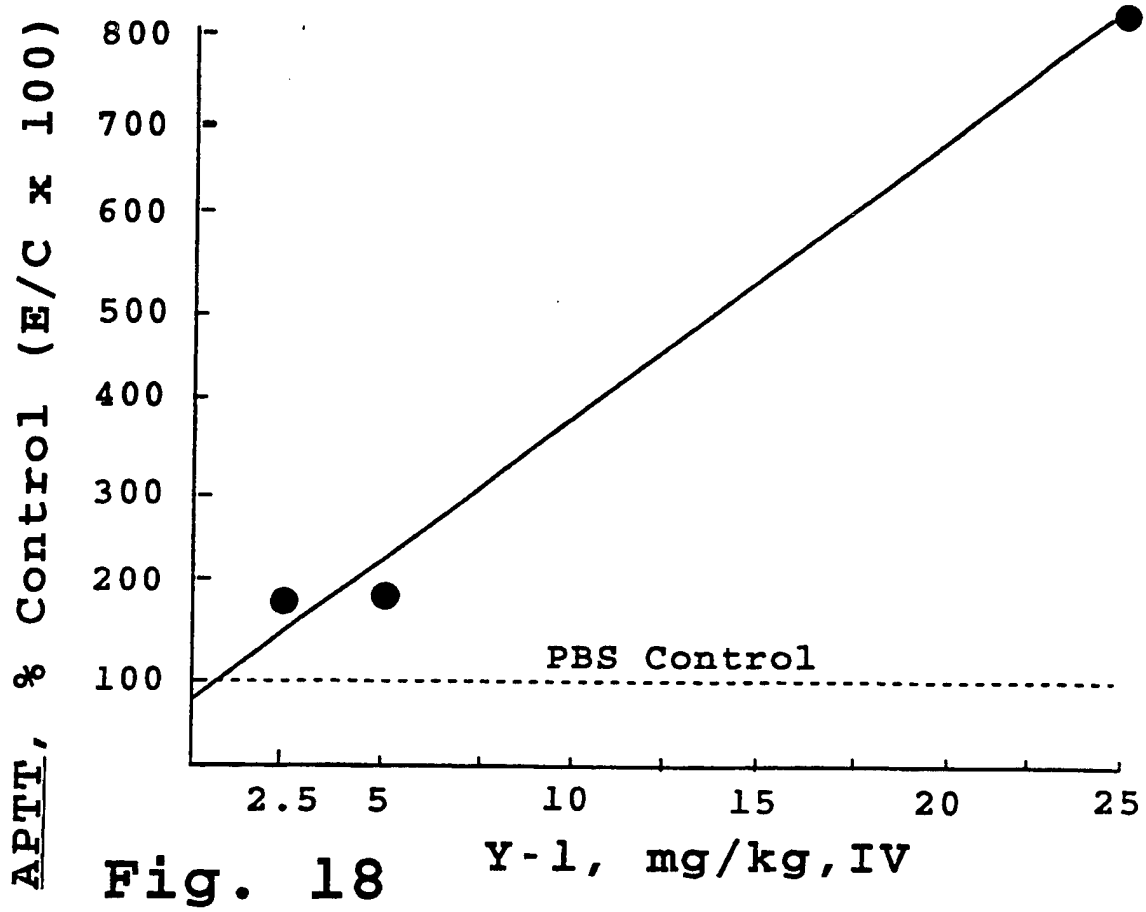

APTT, % Control (E/C x 100)
800
700
600
500
400
300
200
100
PBS Control
2.5
5
10
15
20
25
Y-1, mg/kg, IV

Fig. 18

Fig. 19

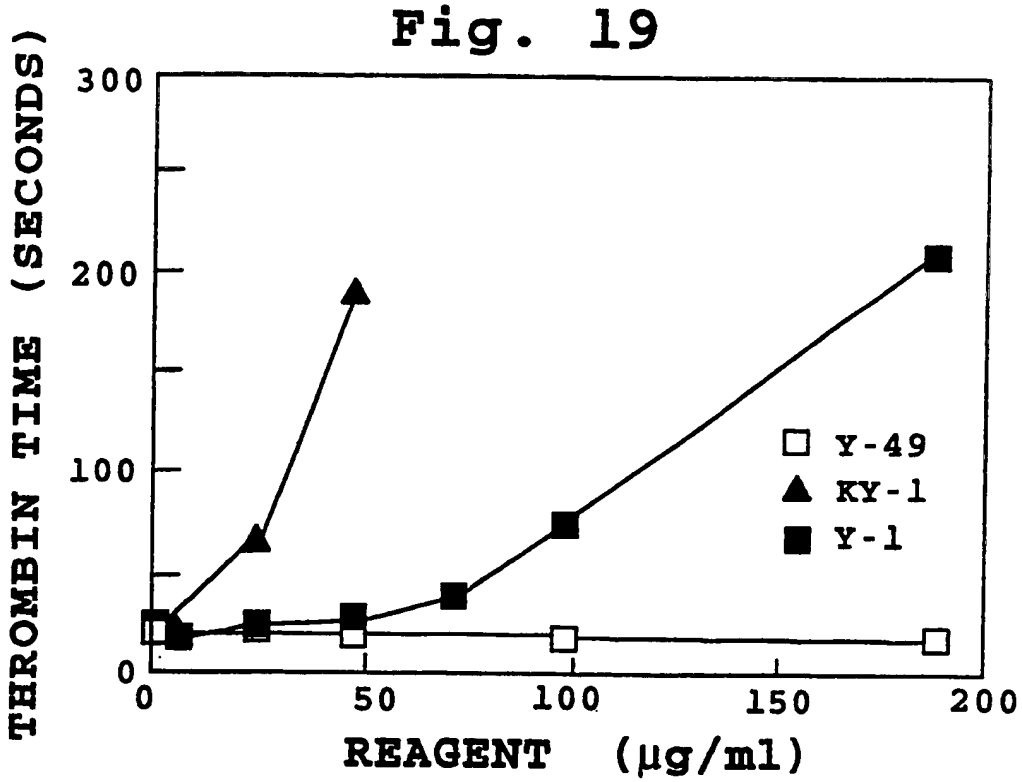

THROMBIN TIME (SECONDS)
300
200
100
0
0
50
100
150
200
REAGENT (μg/ml)
Y-49
KY-1
Y-1

Fig. 20

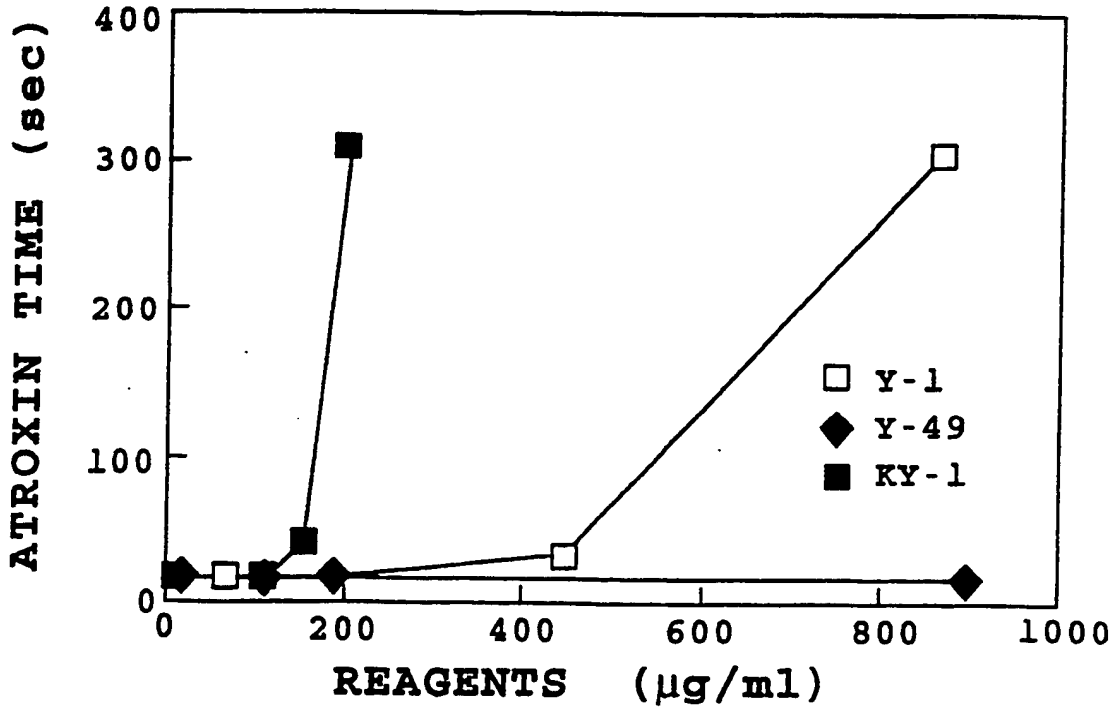

ATROXIN TIME (sec)
400
300
200
100
0
0
200
400
600
800
1000
REAGENTS (μg/ml)
Y-1
Y-49
KY-1

Fig. 21C

Fig. 21B

Fig. 21A

Fig. 21D

Fig. 21E

Fig. 21F

Fig. 21G

Fig. 22A

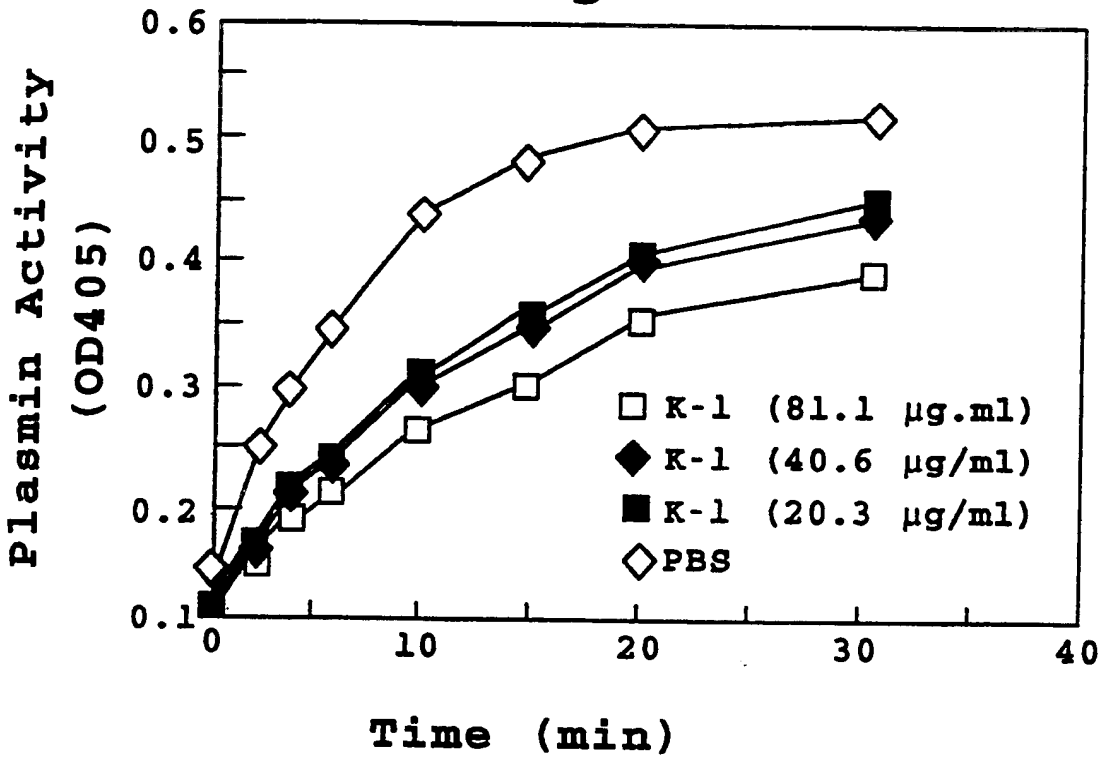
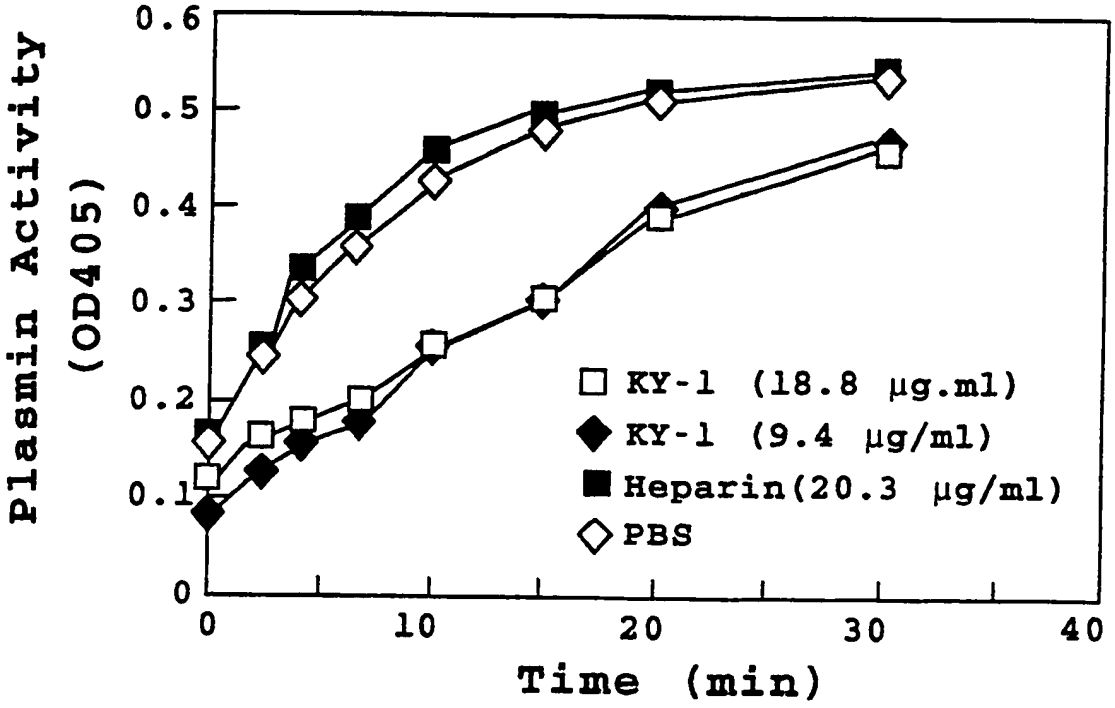


Fig. 22B



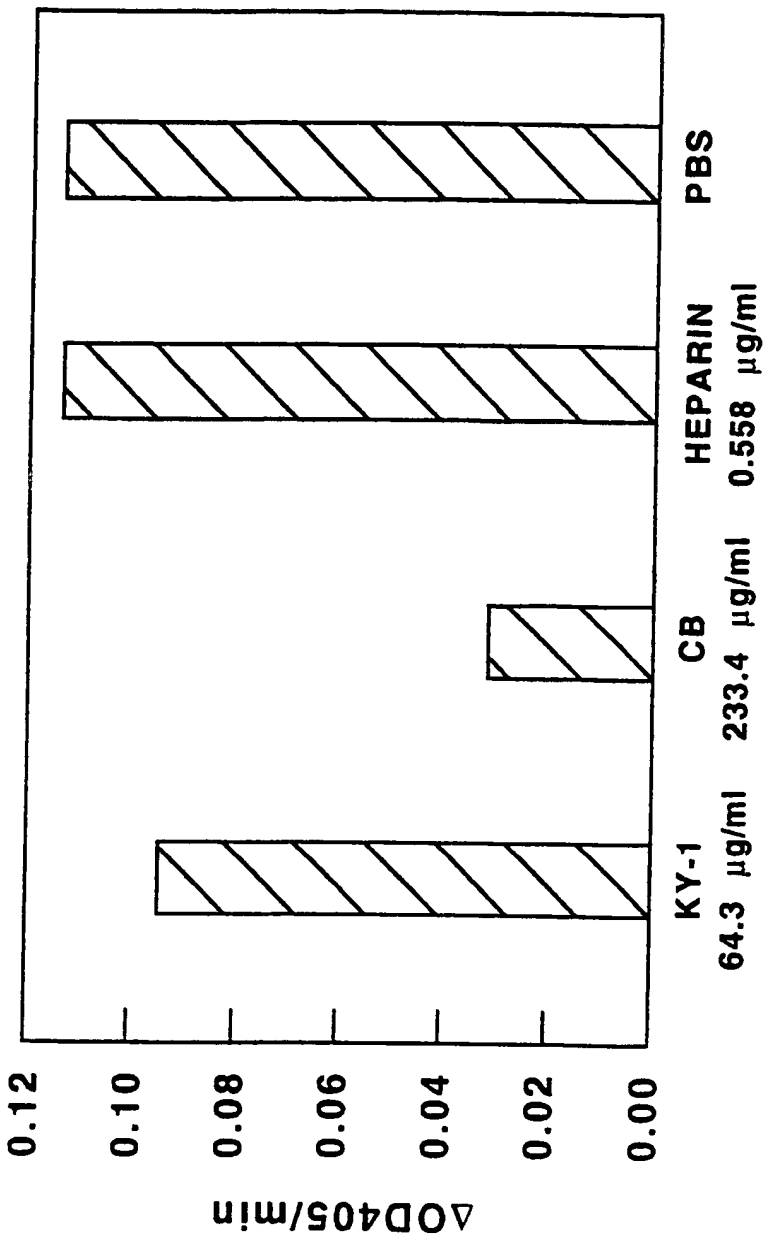

0.12
0.10
0.08
0.06
0.04
0.02
0.00
ΔOD405/min
KY-1
64.3 μg/ml
CB
233.4 μg/ml
HEPARIN
0.558 μg/ml
PBS
REAGENT

Fig. 22C

Fig. 23A

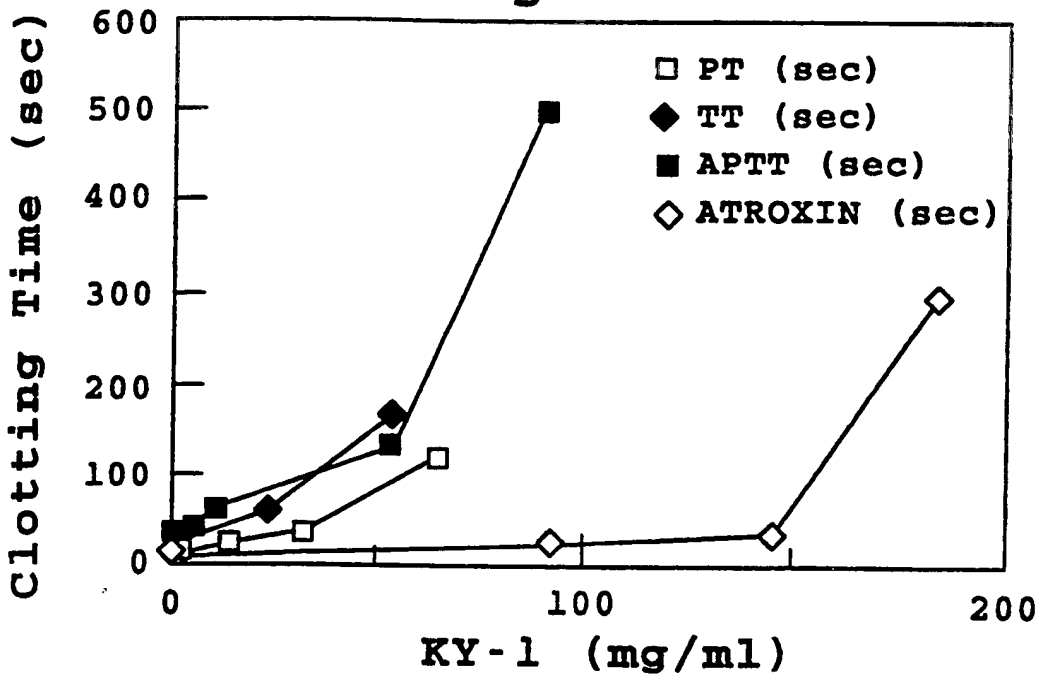


Fig. 23B

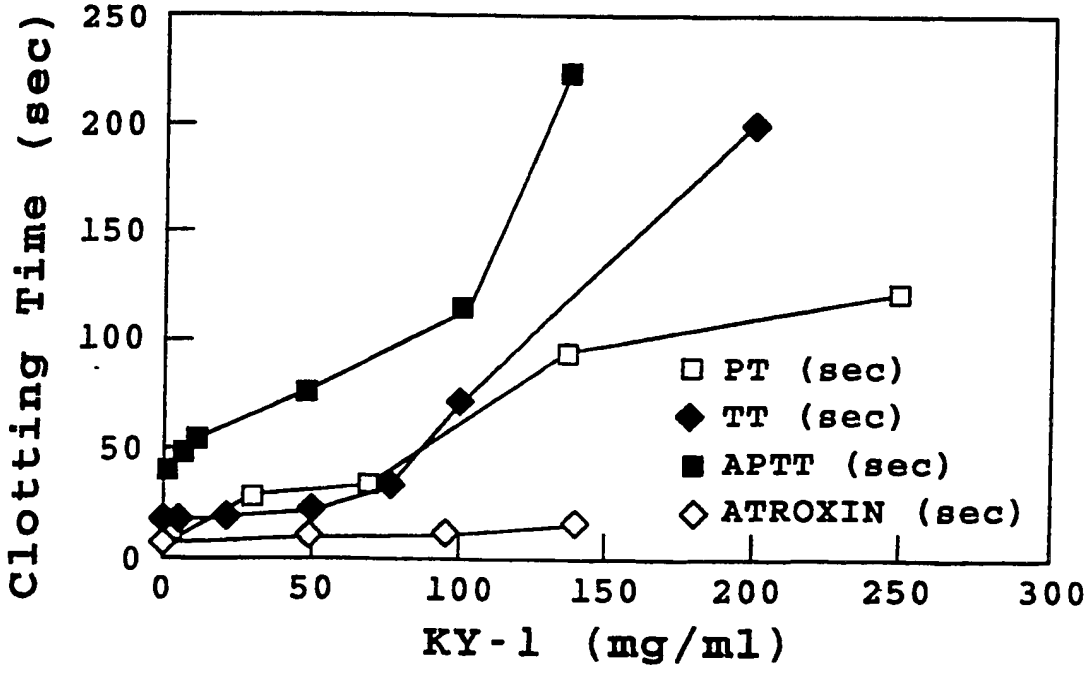


Fig. 24A

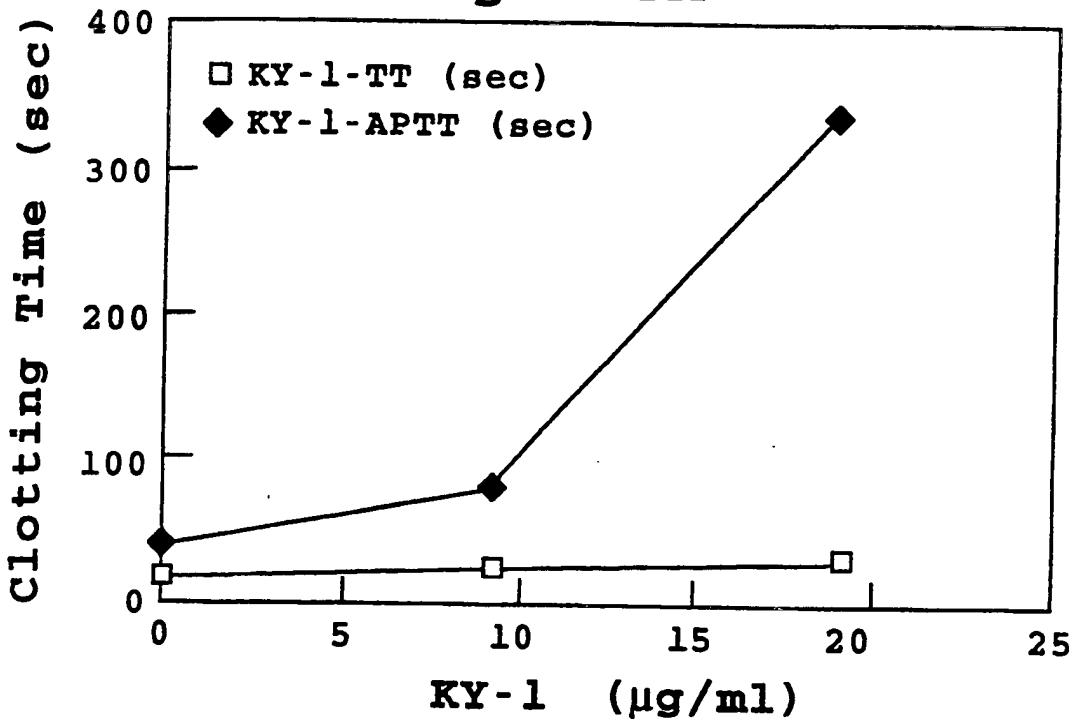
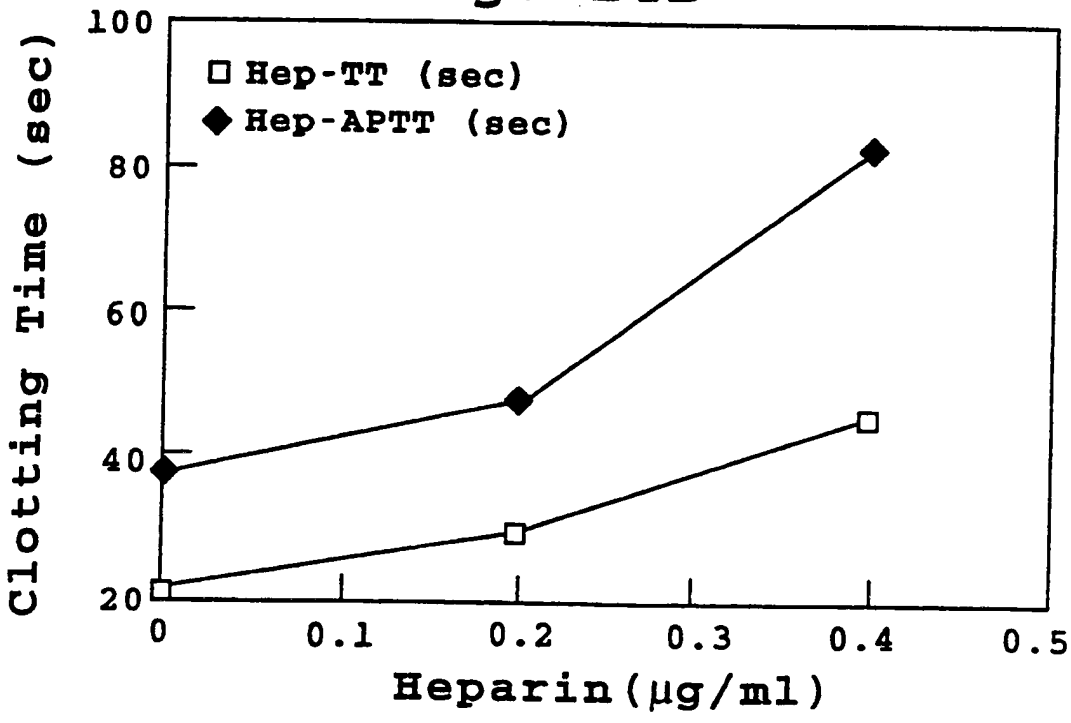


Fig. 24B



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 92/00501

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 31/18 A 61 K 31/185 A 61 K 31/19
A 61 K 31/255 A 61 K 31/215 A 61 K 31/795 C 07 C 309/43
C 07 C 309/44 C 07 C 313/04 C 07 C 311/16 C 07 C 317/22

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K C 07 C

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	Tetrahedron, vol. 46, no. 10, 1990, B.-L. POH et al.: "Complexations of amines with water-soluble cyclotetrachromotropylene", pages 3651-3658, see the whole document ---	1-6,11-14
A	Tetrahedron, vol. 46, no. 17, 1990, B.-L. POH et al.: "Transport of aromatic hydrocarbons by cyclotetrachromotropylene in water", pages 6155-6160, see the whole document ---	1-6,11-14
A	Tetrahedron Letters, vol. 30, no. 8, 1989, B.-L. POH et al.: "A water-soluble cyclic tetramer from reacting chromotropic acid with formaldeyhde", pages 1005-1008, see the whole document --- -/-	1-6,11-14

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17-04-1992	10. 07. 92
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Tetrahedron, vol. 46, no. 12, 1990, B.-L. POH et al.: "Complexations of metal cations with cyclotetrachromotropylene in water and methanol", pages 4379-4386, see the whole document	1-6,11-14
A	Tetrahedron Letters, vol. 31, no. 13, 1990, B.-L. POH et al.: "Complexation of aromatic hydrocarbons with cyclotetrachromotropylene in aqueous solution", pages 1911-1914, see the whole document	1-6,11-14
A	EP,A,0354818 (HADASSAH MEDICAL ORGANIZATION) 14 February 1990, see the whole document	1-6,11-14
A	Tetrahedron Letters, vol. 25, no. 46, 1984, S. SHINKAI et al.: "New water-soluble host molecules derived from calix[6]arene", pages 5315-5318, see the whole document	7-14
X		15
A	J. Am. Chem. Soc., vol. 108, 1986, S. SHINKAI et al.: "Hexasulfonated calix[6]arene derivatives: A new class of catalysts, surfactants, and host molecules", pages 2409-2416, see the whole document	7-14
X		15-16
A	Chemical Abstracts, vol. 105, 1986, (Columbus, Ohio, US), see page 568, abstract no. 208622y, & JP,A,6183156 (SUGAI CHEMICAL INDUSTRY CO., LTD) 26 April 1986, see the abstract	7-14
X		15-16
A	Chemical Abstracts, vol. 113, 1990, (Columbus, Ohio, US), S. SHINKAI et al.: "Synthesis and inclusion properties of neutral water-soluble calixarenes", see page 666, abstract no. 97166w, & BULL. CHEM. SOC. JPN. 1990, 63(4), 1272-4, see the abstract	7-14,17
X		15-16

-/-

III. DOCUMENTS CONSIDERED TO [redacted]ELEVANT (CONTINUED FROM THE SECO[redacted]EET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Chemical Abstracts, vol. 109, no. 19, 7 November 1988, (Columbus, Ohio, US), S. SHINKAI et al.: "Selective adsorption of uranyl ion (UO22+) to a polymer resin immobilizing calixarene-based uranophiles", see page 35, abstract no. 171425r, & J. POLYM. SCI., PART C: POLYM. LETT. 1988, 26(9), 391-6, see the abstract	15,17

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-3, 11-14
because they relate to subject matter not required to be searched by this Authority, namely:
IN VIEW OF THE LARGE NUMBER OF COMPOUNDS WHICH ARE DEFINED BY THE CLAIMS 1-3, 11-14
THE SEARCH WAS LIMITED TO THE COMPOUNDS MENTIONED IN THE CLAIMS 4-10, 15-17.
SEE PCT ART. 6; GUIDELINES..PART B, CHAPT. II.7 LAST SENTENCE AND CHAPT. III, 3-7.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9200501
SA 56552

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 01/07/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0354818	14-02-90	EP-A- 0354714	14-02-90
		JP-A- 2073019	13-03-90
		JP-A- 2256610	17-10-90